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Phosphorylation of Mouse Ascites Tumor Cell Lysine-Rich Histone*

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ABSTRACT: The lysine-rich histone of several mouse tissues has been compared by polyacrylamide electrophoresis to that of mouse Ehrlich ascites tumor cells. A much greater degree of heterogeneity was evidenced in the tumor histones. That a significant portion of this heterogeneity is caused by phosphorylation was demonstrated by labeling the lysine-rich histone with ^{32}P.

This observation was further supported by a reduction in heterogeneity upon removal of the phosphate with alkaline phosphatase.

Johns has shown that mammalian histones can be separated into five major groups by chemical fractionation procedures (Johns, 1964; Phillips and Johns, 1965). Using these separation procedures, a number of workers have shown that the lysine-rich histone fraction contains phosphate groups bound to histone in the form of serine phosphate (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966; Stevely and Stocken, 1968a,b; Marushige *et al.*, 1969). What fraction of the lysine-rich histone is so phosphorylated is not clear, and there is some controversy as to whether the extent of phosphorylation of histone is negatively (Guttierrez and Hnilica, 1967) or positively (Stevely and Stocken, 1968; Buckingham and Stocken, 1969) correlated with the rate of cell division. However, most authors are agreed that it varies from tissue to tissue, and it seems likely that phosphorylation is not necessarily an all-or-none affair, thus opening the possibility for a histone and phosphorylated-histone heterogeneity. Recently, we have described an electrophoretic technique capable of resolving all five histone groups (Panyim and Chalkley, 1969a,b). Upon close examination of the bands, particularly after lengthy electrophoresis, it was shown that several of the bands were made up of multiple components. Further, it is likely that all members of a given electrophoretic group have similar chemistry since they are coisolated during the Johns chemical extraction procedures. A considerable heterogeneity within the lysine-rich histone group has also been documented by other workers (Bustin and Cole, 1969). This raised the possibility that the multiple electrophoretic bands within a group were, in fact, a demonstration of microheterogeneity imposed upon a parent histone molecule by minor modifications of the parent protein with attendant changes in electrophoretic mobility. The most rapidly moving arginine-rich histone is found as an equal-intensity electrophoretic doublet in calf thymus histone and almost exclusively (95%) as a single band for pea histone, which compares very well with the observation of DeLange *et al.* (1969) who found by direct amino acid sequence analysis that 50% of this histone was acetylated in calf thymus and only 6% acetylated in pea histone. In view of the reports of the phosphorylation of lysine-rich histones, it seemed likely that the multiplicity of bands observed in this region was due to varying degrees of phosphorylation of a lysine-rich histone molecule.

This paper presents the results of experiments designed to test this idea, arguing that it is, at least in part, correct. Further, a correlation between the replicative activity of the tissue and the extent of heterogeneity of the lysine-rich histone is described. Slowly replicating tissue, such as liver, contains lysine-rich histone primarily in the nonphosphorylated form, whereas a mouse ascites tumor cell line shows multiple phosphorylation.

Materials and Methods

The strain of Ehrlich ascites tumor cells was propagated in the peritoneal cavity of female mice, strain Swiss Webster. Tumor cells were withdrawn by syringe and washed in 0.9% NaCl, pH 8.0.

[^{32}P]Phosphoric acid (HCl-free) was purchased from New England Nuclear Corp. It was diluted to 1 mCi/ml with 0.9% NaCl–0.01 M Tris (pH 8.0).

^{32}P injections were always 200 μCi/mouse, *i.e.*, 0.2 ml and always intraperitoneal. Injections were given at zero time and 12 hr later. The mice were sacrificed 18 hr after the first injection and tissue was collected.

The isolation and electrophoresis of histone followed methods previously described (Panyim and Chalkley, 1969a).

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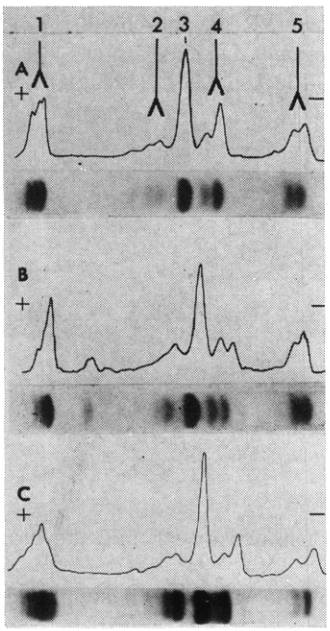

FIGURE 1: Polyacrylamide electrophoresis with microdensitometer traces of mouse tissue histones. The gels, 25 cm long, were electrophoresed at 190 V for 18 hr in 2.5 M urea–0.9 N acetic acid; (A) mouse thymus; (B) mouse liver; (C) Ehrlich ascites tumor cells. The nomenclature is that of Panyim and Chalkley (1969a).

After destaining, the polyacrylamide gels were scanned in a microdensitometer (Gilford, Model 2000), and the scans were then analyzed on a DuPont electronic curve resolver. A standard curve of electrophoresed histone concentration *vs.* peak area was then utilized to determine the amount of histone present in any peak of a microdensitometer scan.

In order to count ^{32}P-labeled histones occurring as specific bands in polyacrylamide gels, the gels were first frozen in Dry Ice–acetone and sliced with a dissecting razor blade. The slices were placed on aluminum planchets, dried at 80–90° for 1–4 hr, and counted on a Nuclear-Chicago Biospan planchet counter, Model 4338.

Alkaline phosphatase (*Escherichia coli*, BAPC, Worthington) was incubated with the lysine-rich histone at 1 mole of enzyme per 4–10 moles of lysine-rich histone. All incubations were in 0.01 M Tris, pH 8.0, at 37°. Incubations were terminated by exhaustive dialysis against cold 0.9 N acetic acid.

Results

Microheterogeneity of Histones of Different Tissues. Histones were isolated from mouse liver, thymus, and mouse ascites tumor cells and electrophoresed through long polyacrylamide gels. The band patterns obtained are shown in Figure 1. Microheterogeneity is seen in several of the major band groups of histone, and in general such heterogeneity is similar from tissue to tissue. However one group, the lysine-rich fraction, shows a tissue-dependent microheterogeneity. The lysine-rich histone from ascites tumor cells shows a greater degree of heterogeneity than we have observed in any other vertebrate system. That all the bands in the lysine-rich region are, in fact, lysine-rich histone is shown by the observation that all the material which resolves into these bands is soluble in 5% trichloroacetic acid or in 5% perchloric acid, a property displayed only by this one group of histones.

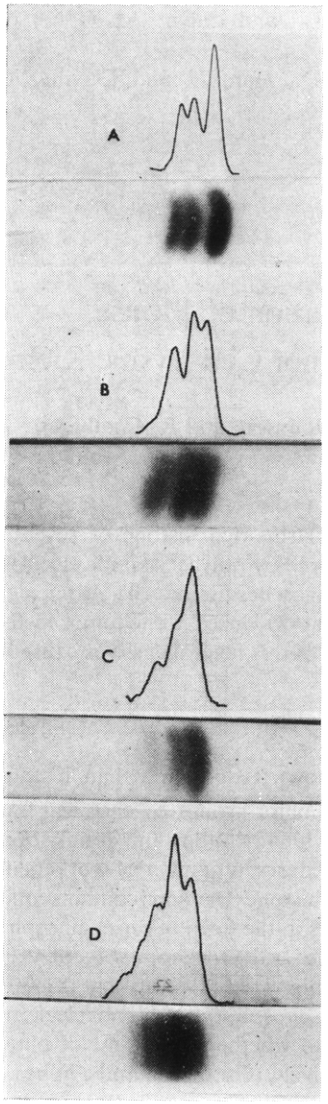

FIGURE 2: High-resolution polyacrylamide electrophoresis with microdensitometer traces of lysine-rich histone. Electrophoresis was on 25-cm gels in 2.5 M urea–0.9 N acetic acid at 190 V for 24 hr. The bands had electrophoresed some 22–23 cm: (A) calf thymus; (B) mouse thymus; (C) mouse liver; (D) Ehrlich ascites tumor cells.

A number of workers have previously compared the histones of tumor and somatic cells (Desai *et al.*, 1969; Hnilica *et al.*, 1962). The most generally accepted conclusion is that they are indistinguishable, at least within the limits of the techniques used for their analysis. The observation that there are, in fact, differences in the extent of heterogeneity of the lysine-rich fraction not previously detected is a result of the very high resolution of which this method is capable.

The histone samples were subsequently electrophoresed on gels similar to those in Figure 1, but for longer periods, so that the faster moving components were eluted from the gel. In this way, we were able to maximally resolve the slower migrating lysine-rich histone. In these experiments, the lysine-rich histone had been electrophoresed for some 25 cm. The data from a comparison study of the three mouse tissues, together with calf thymus, are shown in Figure 2. It is clear that

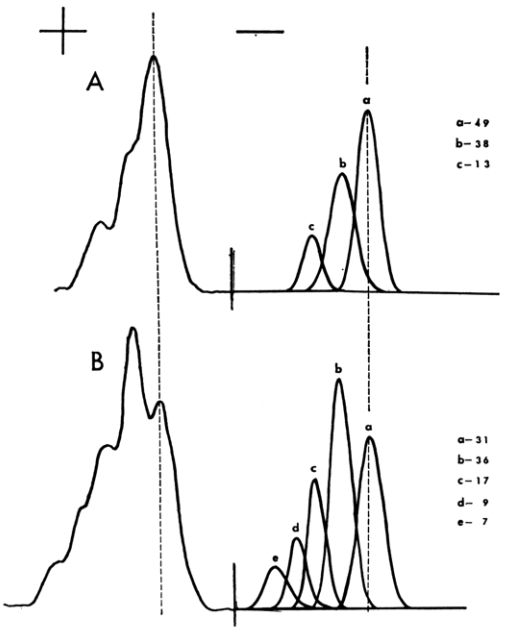

FIGURE 3: Microdensitometer traces and curve analysis of polyacrylamide electrophoresis of lysine-rich histone. The total area under the microdensitometer trace of the lysine-rich band system was assigned an area of 100. Each component peak is then expressed as a percentage of the total area: (A) mouse liver; (B) Ehrlich ascites tumor cells.

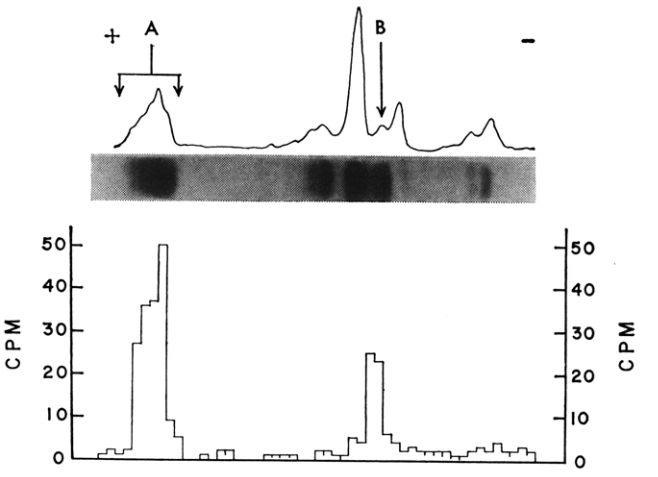

FIGURE 4: Polyacrylamide gel, microdensitometer scan, and ^{32}P incorporation data of Ehrlich ascites tumor cell histone. A and B indicate the two regions containing radioactive label. The counts were obtained from two gels. Electrophoresis was on a 25-cm gel, 2.5 M urea–0.9 N acetic acid for 18 hr at 190 V.

in histones from all these tissues, there are several lysine-rich bands of comparable mobility. The two fastest moving bands are present in all cases; more of the slower moving bands are present in the more actively replicating tissues. Histones from the tumor cell line are unusual in that the most intense band is the second fastest moving, rather than the fastest moving band as found in essentially all other normal tissues, though the actively replicating mouse thymus may be an exception to this rule. It is possible to quantitate the amount of histone in each band by analyzing the densitometer pattern of the bands using a DuPont curve analyzer. Such resolved curves and their relative areas are shown in Figure 3.

Incorporation of ^{32}P into Tumor Histones. Since phosphorylation of serine residues of the lysine-rich histone group has been reported (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966; Stevely and Stocken, 1968a,b; Marushige *et al.*, 1969), it seemed not unlikely that the equispaced bands within this electrophoretic group might be due, at least in part, to phosphorylation of a parent protein, the fastest moving lysine-rich band. To test this hypothesis, and also to examine other histone fractions for phosphorylation, mouse ascites tumor cells were grown in the presence of ^{32}P (sodium phosphate), and the histones were isolated in the usual manner. Electrophoresis of the histone provides a good means for a final purification as evidenced by the low background in the gels. Cutting equal-sized slices from all the bands permits us to locate radioactivity unequivocally.

Radioactivity is found in two of the five electrophoretic groups of histone. The other three groups show no radioactivity. Those bands containing radioactivity are shown in Figure 4. The amount of histone in each radioactive band was computed as described in Materials and Methods, and the amount of radioactivity present in each band was measured after cutting the gel.

The data obtained in this manner are shown in Table I, which contains typical information from one of four separate experiments. The relative amounts of the subbands of the lysine-rich fraction are comparable in separate experiments. The specific activity of ^{32}P incorporation is expressed in terms of cpm/mg of histone. It can be seen that in each experiment the ratios of specific activity for the lysine-rich bands follow a definite trend, with the more slowly migrating fractions having the highest specific activity for the incorporation of labeled phosphate.

The standardized, mean specific activity ratios from four experiments analogous to that described above are summarized in Table II.

Table III presents data for the radioactive labeling of the second fastest histone electrophoretic group (F2a2 in the nomenclature of Johns).

That the ^{32}P label is not simply adhering to the histone molecules was shown by mixing histones with a large excess of labeled orthophosphate and electrophoresing in the usual manner. No labeled material is carried with the histone bands.

The tumor cell line is rapidly replicating, and we wondered whether the level of phosphorylation would be different in the absence of cell division. The phosphorylation of the lysine-

TABLE I: Incorporation of ^{32}P into Lysine-Rich Histone.[a]

Band	cpm/mg
a	327
b	907
c	1571
d	2120
e	2389

[a] Specific activity in cpm/mg for each band of lysine-rich Ehrlich ascites tumor cell histone. For band designation, refer to Figure 3B.

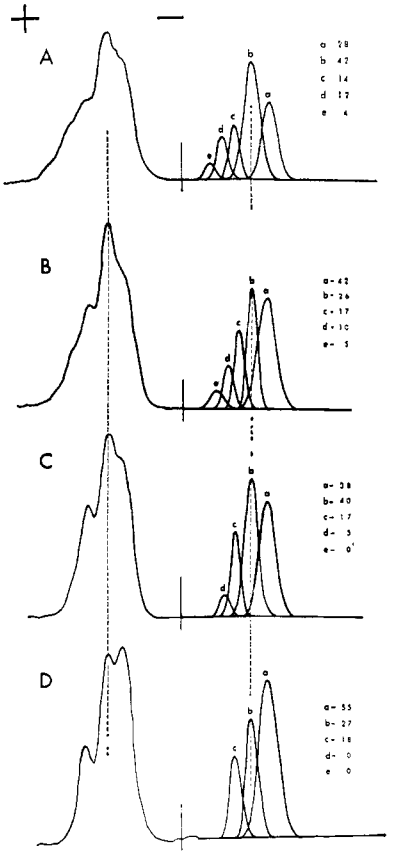

FIGURE 5: Microdensitometer traces and curve analysis from high resolution polyacrylamide electrophoresis of phosphatase treated lysine-rich histone from Ehrlich ascites tumor cells. The total area under the microdensitometer trace of the lysine-rich band region was set equal to 100 and each component peak expressed as a percentage of the total area. Phosphatase was added at 1 mole/4 moles of lysine-rich histone. Incubation was at 37° in 0.01 Tris, pH 8.0, for the time periods reported below: (A) control; (B) 0.5 hr; (C) 12 hr; (D) 32 hr.

rich histone group was studied in mouse thymus, which has a moderately high mitotic index, and also in mouse liver, which has an exceedingly low level of mitosis (only 1 cell in 20,000 is dividing). Although acid-extracted liver histones in bulk show the presence of ^{32}P label (in agreement with previous reports; Ord and Stocken, 1966), upon running the electrophoretic separation we have found that the label is not associated with histone. This, of course, does not mean that liver lysine-rich histones do not contain phosphate groups but rather implies that there is no turnover of such in this system. The more rapidly dividing thymus cells show a low incorporation of ^{32}P into the same electrophoretic groups as found for ascites cells. Again, as for liver histones, direct extraction of lysine-rich histone by the method of Johns indicates the presence of more ^{32}P than can be accounted for by that found as ^{32}P actually bound to histone bands. Much of this additional label, in fact, has an overall negative charge and is ascribed to impurity.

Enzymic Removal of Phosphate Residues from Lysine-Rich Histone. The above data support the notion of multiple, and variable, phosphorylation of a parent lysine-rich histone. If this is correct, we would expect that dephosphorylation should remove the multiplicity of the lysine-rich histone, giving rise to a single band of mobility equal to that of the fastest moving, unphosphorylated parent protein.

The lysine-rich histone from ascites tumor cells was incubated with alkaline phosphatase at 37° and subsequently analyzed for changes in band multiplicity and for the amount of ^{32}P remaining bound to the histone. The time course, showing the band patterns obtained from ascites histone during phosphatase treatment, is shown in Figure 5, and the ^{32}P

TABLE III: Specific Activities of Those Histone Fractions Labeled with ^{32}P.[a]

Region Indicated in Figure 4	cpm/mg
A	2532
B	1652

[a] Specific activities of the two ^{32}P-labeled histone regions from Ehrlich ascites tumor cells. The counts were obtained by pooling slices from two polyacrylamide gels. The areas sliced and counted are indicated in Figure 4.

TABLE II: Standardized Specific Activity of Labeled Histone Electrophoretic Bands from Four Experiments.[a]

Band	Average cpm/mg
a	0.27 ± 0.14[b]
b	1.00 definition
c	1.58 ± 0.53
d	2.31 ± 0.76
e	2.57 ± 0.67

[a] Average specific activities from four experiments for the five bands of lysine-rich histone from Ehrlich ascites tumor cells. The specific activity of band b was set equal to 1.0, and all other bands were then expressed relative to band b. [b] Standard deviation.

TABLE IV: Effect of Phosphatase Treatment on ^{32}P-Labeled Lysine-Rich Histone.[a]

Band	Control (without Phosphatase) cpm/mg	Phosphatase Treated cpm/mg
a	647	244
b	820	487
c	1260	308
d	2830	0
e	2833	0

[a] Specific activities for the five bands of Ehrlich ascites tumor cell histones with and without alkaline phosphatase treatment. Alkaline phosphatase treatment was carried out at pH 8.0. For band designation, see Figure 3B.

release data are presented in Table IV. It is seen that extended phosphatase treatment removes about 80% of the ^{32}P from the lysine-rich histone, and at the same time, the initial five-band heterogeneity is converted into a three-band heterogeneity. Control incubations in the absence of enzyme did not indicate either change in the five-band heterogeneity or a release of bound ^{32}P. The remaining 20% of the label has proved very resistant to hydrolysis and more experimentation is required, but it seems unlikely that removal of this residual amount of orthophosphate will give rise to a single parent lysine-rich histone.

Discussion

We have shown that the electrophoretic heterogeneity of two of the five major mammalian histones is due, at least in part, to phosphorylation of histones. However, for the lysine-rich histone fraction of ascites tumor cells, it is clear that phosphorylation is not the sole contributor to electrophoretic heterogeneity. It is possible that the three remaining bands, after phosphatase treatment, represent three different lysine-rich polypeptides (Bustin and Cole, 1969) which would compare well with the system observed in pea lysine-rich histones. On the other hand, there remains the possibility that these three bands are a result of charge change such as acetylation[1] in a single parent polypeptide. This system is easily capable of detecting unit changes in the charge on a polypeptide, and the extent of the spacing between the bands argues for differences in charge of this order of magnitude.

We are now in a position to describe the histone complement of a typical mammalian somatic nucleus. There are five different histone molecules. Four of these molecules may be modified, giving rise to microheterogeneity (as detected electrophoretically) without changes in the primary sequence. Two are modified by phosphorylation; one, and possibly two, are modified by acetylation. The remaining histone molecule (F2b) has not so far been found to exhibit microheterogeneity. It is interesting to note that this fraction, and the lysine-rich histone, were the most extensively phosphorylated in the *in vitro* experiments using isolated histone fractions (Langan, 1968; Meisler and Langan, 1969). The microheterogeneity we describe is only that which involves a change in the charge on a given protein. Other forms of microheterogeneity which have been described as occurring in histones (Kim and Paik, 1965; Elgin *et al.*, 1970) would not be detected in this system. This discussion is restricted to those changes which we can detect and measure in the electrophoretic system. With the exception of the lysine-rich histone group, we have observed that all tissues examined contain the same derivative species of a given histone, though in varying amounts. The extent of phosphorylation of the lysine-rich histone, however, is highly tissue dependent, being more extensive in more rapidly dividing tissues.

The significance of the demonstration of microheterogeneity of certain histone groups in understanding the role of histone in the somatic cell is not immediately apparent. However, it has recently been shown that the positive charge in the lysine-rich histone is asymmetrically distributed (Bustin *et al.*, 1969). One-half of the molecule has very few positive charges, and if present in this half of the molecule, the effect of several phosphate groups at physiological pH would be to electrostatically neutralize this half of the molecule, perhaps permitting the molecule to act as a switch having one conformation (and interaction) in the nonphosphorylated form and a different conformation when phosphorylated. Since there appears to be relatively little new phosphorylation in the nondividing somatic cell, it seems unlikely that this is used as a general mechanism for modifying gene expression.

[1] A recent determination of the acetyl groups on this histone fraction by Dr. L. Stegink of the Department of Pediatrics and Biochemistry gives a value of 0.4 mole of acetate per mole of lysine-rich histone.

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