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DIFFERENTIAL PHOSPHORYLATION OF HISTONE H1 SUBFRACTIONS IN VIVO

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SUMMARY: A study was made of the phosphorylation of chromatographically purified histone H1 subfractions from the liver of premetamorphic tadpoles (Rana catesbeiana). Two H1 subfractions were obtained which differed in terms of net incorporation of [^{32}P]phosphate in vivo. Analysis of N-bromosuccinimide cleavage products further revealed that the two subfractions also differed in the relative distribution of [^{32}P]phosphate in N- and C-terminal regions of the molecule. Incorporation of [^{32}P]phosphate into both regions of the molecule occurred virtually exclusively in serine residues.

INTRODUCTION

Since regulation of gene expression in eukaryotes is considered to be at least partially dependent on some aspects of chromatin structure (1), numerous studies have examined chemical modifications, such as phosphorylation, of a well-defined class of chromatin structural proteins, the histones, to ascertain whether variations in such modifications may be involved in altered capacity for gene expression. Of the different histone classes, the histone H1 group is notable in that it exhibits the greatest inter- and intraspecies variability in both number and primary sequence of subfractions (2-4). Additionally, changes in phosphorylation of H1 molecules have been observed in conjunction with selective tissue responses to hormones (5-7) and at different phases of the cell cycle (e.g., 7-12). Although in vitro studies have clearly demonstrated variation among H1 subfractions in the potential for phosphorylation at

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specific sites (13), few studies have so far examined phosphorylation of individual H1 subfractions in vivo.

This initial characterization of in vivo phosphorylation of H1 subfractions in tadpole liver represents part of a continuing investigation of the structure and function of tadpole liver chromatin (7,14-17). This organ is suitable for such a study since the fractionated histones can be obtained in good yield, and initial characterizations of highly-purified fractions have already been performed (7,17). Moreover, tadpole liver contains only two H1 subfractions which can be easily resolved. The results to be presented here demonstrate distinct differences in the in vivo phosphorylation pattern of tadpole H1 subfractions.

METHODS

Premetamorphic Rana catesbeiana tadpoles were obtained from Mogul-Ed Corp. (Oshkosh, Wisconsin) and maintained at 15° in running dechlorinated tap water. Groups of 15-20 animals were equilibrated at 23° for 2-3 days and intraperitoneally injected with carrier-free (^{32}P]orthophosphate (New England Nuclear) at a dosage of approximately 100 μCi/gm body weight. After 5 hours livers were excised, and nuclei and histones were isolated as described previously (17), except that the corresponding sucrose buffers used in nuclear isolation were composed as follows: (a) 0.33 M sucrose-0.1 M citric acid-25 mM NaF; (b) 1.9 M sucrose-0.025 M citric acid-3 mM $CaCl_2$. This modification of the earlier method gave essentially quantitative yields of nuclei, based on DNA recovery. Net incorporation of phosphate into histones extracted from liver nuclei isolated by either method was essentially identical.

Fractionation of total histones and resolution of H1 subfractions were as previously described (17). Cleavage of histone H1 by N-bromosuccinimide (Pierce Chemical Co.) was performed according to Bustin and Cole (18). Subsequent chromatography on Sephadex G-100 (1.2 x 125 cm) was according to Rall and Cole (19). The procedures of Hohmann et al. (20) were used to identify phosphoserine and phosphothreonine. Unlabeled phosphoserine and phosphothreonine standards were from Sigma. Autoradiography on Kodak XR-5 film was carried out at -80° using a Cronex Quanta-III (Dupont) intensifying screen (21).

Determinations of the specific radioactivity of inorganic phosphate pools were carried out on supernatants of 1 N perchloric acid extracts of liver homogenates within 45-60 minutes after homogenization. Inorganic phosphate was separated from organic phosphate esters by extraction into n-hexanol as a phosphomolybdate complex (22). Colorimetric determination of phosphate in the hexanol phase was performed at 660 nm according to Martin and Doty (23). Aliquots of the hexanol extract were also taken for Cerenkov counting of ^{32}P (24). All organic and aqueous samples containing ^{32}P were counted by Cerenkov radiation, with an efficiency of approximately 37%.

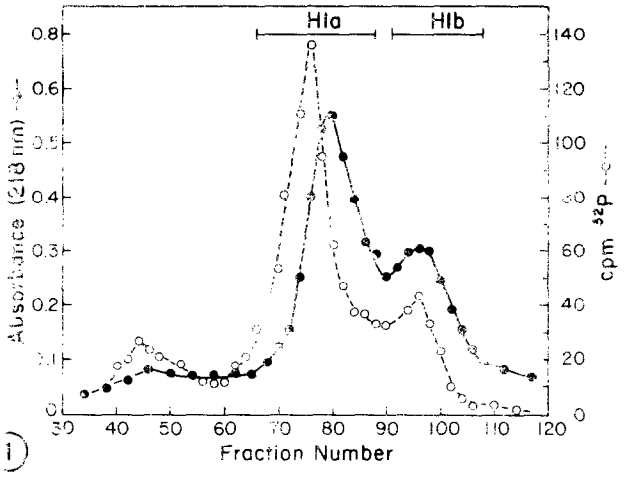
Procedures not described here have been detailed previously (17).

RESULTS AND DISCUSSION

Figure 1 shows the pattern of tadpole liver H1 subfractions obtained by ion-exchange chromatography on Amberlite IRC-50 resin. The same two subfractions can be resolved by electrophoresis on acid-urea polyacrylamide gels and are also distinct molecular species in terms of amino acid composition (not shown). The subfractions obtained from two different lots of animals were pooled as indicated in Figure 1, yielding the results shown in Table I. The net phosphate incorporation into the subfractions during the labeling period, expressed as mmoles Pi/mole H1, has been obtained by dividing the specific radioactivity of the histones by the specific radioactivity of the liver inorganic phosphate pool.

The results shown in Table I reveal a clear difference between subfractions H1a and H1b in terms of net phosphate incorporation. Although differences in the extent of *in vivo* phosphorylation of H1 subfractions have been described by other investigators (e.g., 20,25), this is the first report to present quantitative comparisons of net phosphate incorporation and intramolecular phosphate distribution for *in vivo* labeled H1 subfractions. It remains to be established whether the results reported here are due to differences in numbers of phosphorylated sites, turnover rates of specific phosphate groups, or some combination of the two. The very low levels of phosphorylation and radioactivity in these experiments have so far prevented precise determination of the number of phosphate groups in the individual subfractions. However, a lower limit on the number of phosphorylated sites can be obtained by analysis of H1 fragments derived by specific chemical cleavage.

N-Bromosuccinimide treatment efficiently cleaves H1 into a C-terminal fragment (N1) of about 15,000 daltons and an N-terminal fragment (N2) of about 6,000 daltons (18,19). Analysis of these fragments shows that there are at least 2 phosphorylated istes in both tadpole liver H1 subfractions (Table II). Since these fragments are produced in equimolar amounts, the data have been presented simply as total counts in the N1 and N2 fractions. Here again the



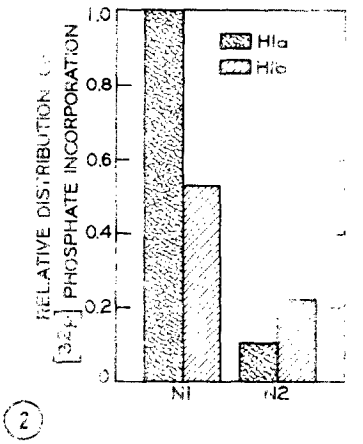


Figure 1. Chromatographic resolution of tadpole liver H1 subfractions on Amberlite IRC-50 (1.2 x 20 cm). Following an initial wash with 7.5% guanidinium chloride buffer (40 ml), H1 subfractions were eluted with a linear 7.5 - 12.5% guanidinium chloride gradient (160 ml total) as described previously (17). Fractions of 0.7 ml were collected at a rate of 2.8 ml/hr. Subfractions a and b were obtained by pooling the fractions indicated by the horizontal bars.

Figure 2. Relative distribution of phosphate incorporation into N1 and N2 regions of tadpole liver H1 subfractions. The results have been derived from data in Tables I and II. Net phosphate incorporation into the N1 region of H1a has arbitrarily been chosen to be 1.0.

TABLE I: INCORPORATION OF [^{32}P]PHOSPHATE INTO HISTONE H1 SUBFRACTIONS OF TADPOLE LIVER IN VIVO.

Expt.	H1 subfraction	cpm ^{32}P / mg H1	mmoles Pi / mole H1	Relative ^{32}P incorporation
I	a	2600	18	1.00
	b	1600	12	0.67
II	a	2930	26	1.00
	b	1930	17	0.65

TABLE II: [^{32}P]PHOSPHATE DISTRIBUTION IN N-BROMOSUCCINIMIDE FRAGMENTS OF TADPOLE LIVER H1 SUBFRACTIONS LABELED IN VIVO.

H1 subfraction	NBS fragment	Total cpm ^{32}P	Relative ^{32}P incorporation
a	N1	931	1.00
	N2	95	0.10
b	N1	154	1.00
	N2	64	0.42

two subfractions are seen to be distinct in terms of relative distribution of phosphate incorporation. Both these subfractions had phosphate distributions different from that of unfractionated tadpole liver H1, with N1/N2 = 1.00/0.22 (data not shown) -- a ratio consistent with the sums of the phosphate contributions of each subfraction. From the data in Tables I and II the relative distribution of the phosphate incorporation into H1a and H1b can be determined for both N- and C-terminal fragments (Fig. 2). Thus, it can be seen that the C-terminal region (N1) of H1a has incorporated twice as much phosphate as that of H1b, while the N-terminal region (N2) of H1a has incorporated only half as much phosphate as that of H1b. However, it should be emphasized that this distribution profile does not necessarily represent the physical distribution of phosphate in an individual H1 molecule, since the data do not distinguish between, e.g., the possibilities of all phosphorylated H1 molecules containing phosphate in both N1 and N2 regions or some H1 molecules phosphorylated only in N1 with others phosphorylated only in N2.

Analysis of N1 and N2 of unfractionated tadpole liver H1 using the method described by Hohmann et al. (20) demonstrated that phosphorylation in both regions of H1 occurred almost exclusively on serine residues. A very faint trace of [^{32}P]phosphothreonine could be detected only in N2 and only after long periods of autoradiography. Since tadpole liver is a stable population of cells with very little proliferation (reviewed in 27), the present results are consistent with the report that phosphorylation of threonine residues is significant only during mitosis (26). It should also be pointed out that all histones used in these studies were isolated at low pH, and therefore phosphorylation at acid-labile sites would not have been detected (28).

Although there are no other comparable reports on the distribution of in vivo phosphate incorporation into individual H1 subfractions, there are descriptions of phosphate incorporation into N1 and N2 regions of unfractionated H1. Depending on the tissue and the phase of the cell cycle, phosphate

incorporation was found to occur only in N1 (26,29), only in N2 (30), or in both N1 and N2 (26,31). The absence of a consistent pattern is not surprising, considering the differences in growth state of the cells studied, as well as possible species variations in turnover rates of individual phosphate groups and sequence location of phosphorylatable sites. The relatively high serine and threonine content found for tadpole H1, compared to calf thymus H1, for example, suggests a greater potential for phosphorylation than in some other organisms. Although high-voltage paper electrophoresis at pH 1.9 of tryptic digests of H1a and H1b revealed different patterns of ^{32}P-labeled peptides (not shown), such an analysis is inconclusive for demonstrating phosphorylation at different sites in the 2 subfractions because of possible ambiguity of tryptic cleavage and the likelihood of sequence variation at tryptic cleavage sites.

The relative complexity of the H1 subfractions of many other organisms makes difficult the interpretation of changes in phosphorylation, especially when the H1 histones are studied as a single group, as is generally the case. Thus, the low number of H1 subfractions in tadpole liver, coupled with their clear difference with regard to phosphorylation, makes this organ highly useful for studying specific H1 phosphorylation sites to gain clues as to their possible functional significance, not only by species comparisons of phosphorylation site sequences, but also by determining the sites of altered phosphorylation in response to hormonal stimulation (7,32).

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