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Regulation of the Phosphorylation of Chromatin-associated Proteins in *Lemna* and *Hordeum*[1]

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ABSTRACT

This paper represents attempts to observe alterations in the pattern of chromatin protein phosphorylation in *Lemna* and barley (*Hordeum vulgare*).

As judged by *in vitro* labeling the phosphorylation profile is substantially altered during germination. This may not be the result of specific tissue differentiation, however, because phosphorylation does not differ markedly between the embryonic root and shoot. Treatment of nuclei from germinating embryos with low concentrations of sodium or potassium chloride produced phosphorylation patterns similar but not identical to those found in nuclei from ungerminated embryos.

Treatment of *Lemna* with abscisic acid *in vivo* causes substantial alterations in the labeling of three protein bands and part of this may be duplicated by labeling isolated nuclei from treated tissue with γ^{32}P-ATP. Some effects of light/dark transition on *Lemna* chromatin protein phosphorylation are also described.

We have previously shown that chromatin-associated proteins of *Lemna perpusilla* and *Hordeum vulgare* can be phosphorylated both *in vivo* and in isolated nuclei preparations (7). Acid-insoluble nonhistone components appeared to be preferentially phosphorylated. From work with animal systems it has been suggested that these nonhistone chromatin proteins may play a major role in the regulation of gene expression in eukaryotic cells (1). Particularly the phosphoprotein components of this protein fraction have many properties expected of specific genetic regulatory molecules (6).

The possible involvement of chromatin phosphoproteins in genomic regulation in plants has not yet been investigated. This paper represents an initial approach to this problem by investigating what parameters may induce alterations in phosphorylation patterns. The two reasons behind this approach are: (*a*) an attempt to locate a means of regulating protein kinase activity in plants; and (*b*) an attempt to throw further light on what are at the present time largely speculative ideas of the function of phosphorylated chromatin proteins. The various developmental and environmental states examined are embryo germination, simple tissue differentiation, effects of abscisic acid, varying periods of darkness, and the effects of low concentrations of K^+ and Na^+.

MATERIALS AND METHODS

Plant Material. *Lemna perpusilla* was cultured and *Hordeum* embryos isolated as previously described (7). Sterile *Hordeum* embryos were germinated at 30 C in the dark on 1.5% (w/v) agar containing the basal mineral medium of Joy and Folkes (2) supplemented with 4% (w/v) casein hydrolysate. Germination times given include the time required for imbibition, isolation, and sterilization of the embryos. *Lemna* cultures subjected to dark treatment were kept in complete darkness at 28 C for periods of up to 72 hr. Growth appeared to be only slightly inhibited by this treatment as the fresh weight of the cultures increased and a substantial number of etiolated fronds were present after this time period.

Protein Kinase Assay. *Hordeum* and *Lemna* nuclei were isolated as described in the previous paper (7), except that a step involving the centrifugation of *Lemna* nuclei into a cushion of 1.78 M sucrose, 10 mM tris, pH 7.4, 5 mM $MgCl_2$ was included. The kinase activity of the nucleus was measured as previously described (7).

Proteins were labeled in isolated nuclei using γ^{32}P-ATP. Chromatin was isolated from these labeled nuclei; labeled proteins were separated by gel electrophoresis (4, 5), stained, scanned, and sliced into 1-mm sections as described in the previous paper (7).

RESULTS

Figure 1 shows nuclear protein kinase assays (Fig. 1c) and *in vitro* chromatin protein phosphorylation patterns observed after germinating barley embryos for 5 and 21 hr (Fig. 1, a, b, and d). As Figure 1c shows, nuclei isolated from 5-hr and 21-hr embryos differ distinctly in the progress curves for total phosphorylation. Addition of casein does not obviate these differences and thus they do not appear to result from changes in the levels of endogenous sustrates during germination. Figure 1, a and b, shows the acid-urea gel phosphorylation patterns of chromatin-associated proteins from the 5-hr and 21-hr germinated embryos. Proteins were labeled by isolating nuclei from embryos of the two ages, incubating them in γ^{32}P-ATP, and then preparing the chromatin proteins from these labeled nuclei. Because the progress curves of the nuclear kinase assay differ between the two embryo ages, incubation times of 10 and 20 min have been included in Figure 1, a and b. Comparison of Figure 1, a and b, reveals certain discrete differences

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in the phosphorylation patterns which are not incubation time-dependent but presumably the result of differences in the two sets of nuclei. The reproducibility of this germination change may be seen by comparing Figures 2 and 3 in the previous

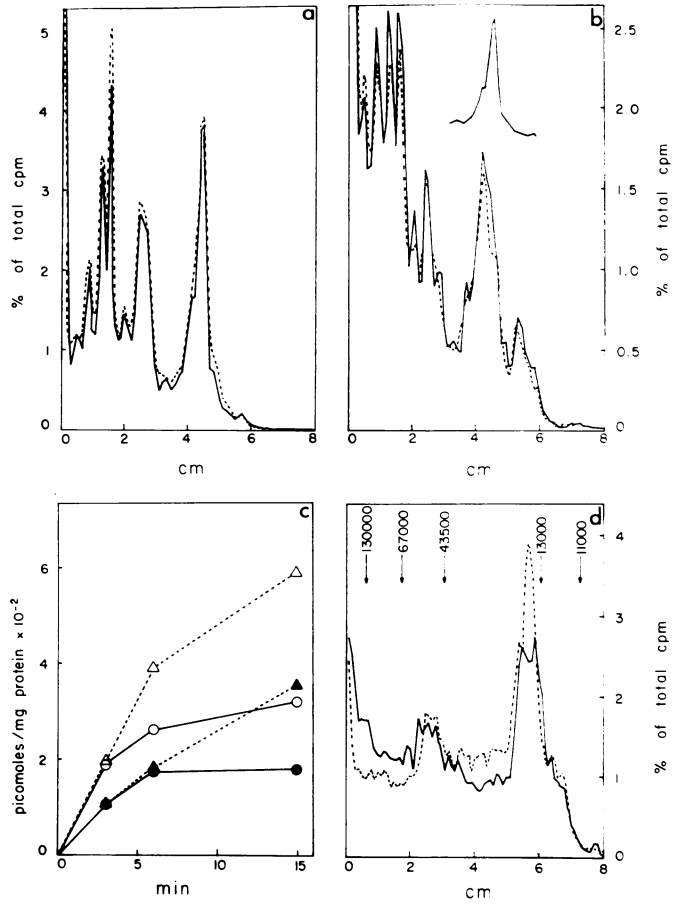

FIG. 1. Protein phosphorylation profiles and protein kinase assays of nuclei isolated from 5-hr and 21-hr germinated barley embryos. a and b: Acid-urea gel electrophoresis profiles of chromatin phosphorylated proteins from 5 hr (a) and 21 hr (b) germinated embryos. Nuclei were isolated from the two batches of embryos and incubated in 50 μCi γ^{32}P-ATP for 10 min (– – –) or 20 min (——). The chromatin proteins were prepared from these labeled nuclei, subjected to gel electrophoresis, and the gels were stained, scanned, and sliced into 1-mm slices and counted. Protein loadings on the gel in each case were about 30 μg, and the total radioactivity on the gel varied from 5×10^4 to 11×10^4 cpm. The ordinate shows the percentage of the total activity on the gel in each individual slice and the abscissa represents the mobility. c: Protein kinase activity of nuclei isolated from barley embryos germinated for 5 hr (△▲) or 21 hr (○●). Nuclei were isolated from the two ages of embryo and incubated in γ^{32}P-ATP. The incorporation of ^{32}P into cold acid-insoluble material was determined as described in the methods section, and this was converted into picomoles of Pi transferred. Open symbols represent kinase activity in the presence of casein (5 mg/ml) and closed symbols in the absence of casein. d. SDS gel electrophoretic profiles of chromatin phosphorylated proteins from 5 hr (– – –) and 21 hr (——) germinated embryos. Nuclei were isolated from these two ages of embryo and incubated for 10 min in γ^{32}P-ATP. Chromatin proteins were prepared, separated by SDS gel electrophoresis, and the gels were stained, scanned, and sliced into 1-mm slices. Protein loadings in both cases were about 60 μg and the radioactivity on the gel varied from 12×10^4 to 16×10^4 cpm. The ordinate shows the percentage of total radioactivity found in each gel slice and the abscissa the mobility.

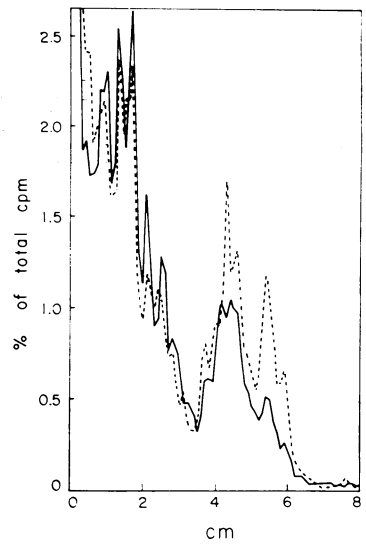

FIG. 2. Acid-urea gel electrophoresis profiles of phosphorylated chromatin proteins isolated from the root (——) or the shoot (– – –) of barley embryos. Embryos were germinated for 21 hr and then divided into root and shoot portions. The nuclei were isolated from these two tissues and incubated for 20 min in γ^{32}P-ATP. Phosphorylated chromatin proteins were prepared, separated by acid-urea gel electrophoresis, and the gels were stained, scanned, and sliced into 1-mm slices. Protein loadings were about 20 μg and approximately 6×10^4 cpm were applied to each gel. The ordinate represents the percentage of the total activity on the gel found in each slice and the abscissa the mobility.

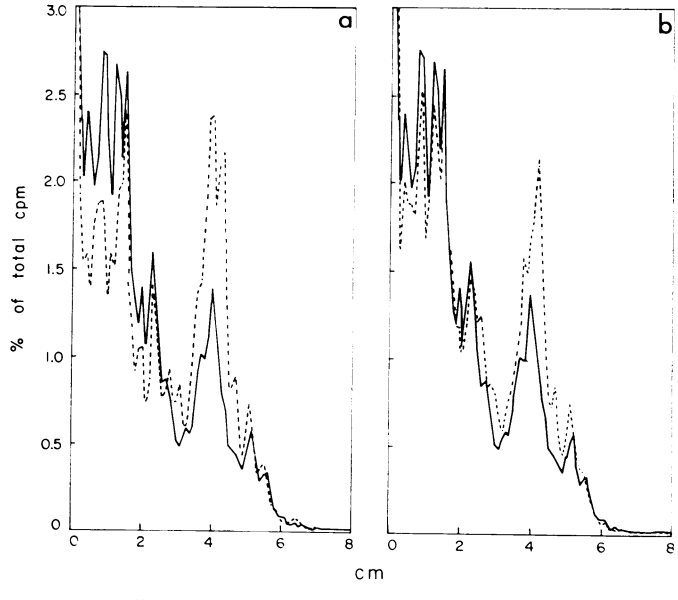

FIG. 3. Effect of low concentrations of KCl or NaCl on the phosphorylation patterns of barley embryo chromatin proteins. Nuclei were isolated from 21-hr germinated embryos, divided into three equal portions, and incubated in 0.3 M sucrose, 0.05 M tris (pH 7.4) 5 mM $MgCl_2$ containing 50 μCi of ^{32}P-ATP for 20 min in the presence of 0.15 M KCl or 0.1 M NaCl, or with no additional salt (control). The chromatin proteins were prepared from these labeled nuclei, separated by acid-urea gel electrophoresis, and the gels were stained, scanned, and sliced into 1-mm slices, and counted. The ordinate represents the percentage of the total activity on the gel found in each slice. Protein loadings for each gel were about 24 μg and the radioactivity on the gel 25×10^3 cpm. a: control (——); 0.15 M KCl (– – –); b: control (——) 0.1 M NaCl (– – –).

paper (7) which are of 5-hr germinated embryos with Figures 2 and 3 in this paper which are of 21-hr germinated embryos.

The inset of Figure 1b shows the same region of the gel from Figure 1a and is designed to show that the fastest running component in this rather broad band, which we have shown to be acid-soluble (7), is decreased about 4-fold during germination. The major qualitative change occurs in the fastest migrating phosphorylated protein which runs at about 5.5 cm in Figure 1b, but is not present in 5-hr germinated embryos (Fig. 1a). This protein migrates with one of the densely staining bands on the gel and may also be a histone. Other less dramatic changes which take place during germination may be seen in the slower migrating region of the gel (0–3 cm) in which some bands are decreased and others increased 2- to 2.5-fold. Figure 1d shows SDS gel profiles of phosphorylated chromatin proteins prepared by incubating nuclei from the two embryo ages for 10 min in labeled ATP. The germination changes, shown in Figure 1, a and b, are severely masked in common with the reduced discrimination provided by this separative method.

After germinating for 21 hr, embryos have been subdivided into root and shoot portions and the nuclei prepared and labeled in order to assess the relative contributions of the two tissue types to the pattern of phosphorylation. Figure 2 shows the phosphorylation profiles obtained and it is obvious that the differences between the phosphorylation patterns were less than those between the germination stages. The main difference between the root and shoot was the higher relative phosphorylation of the bands in the region 4 to 6 cm in the shoot profile, two components of which may be histone.

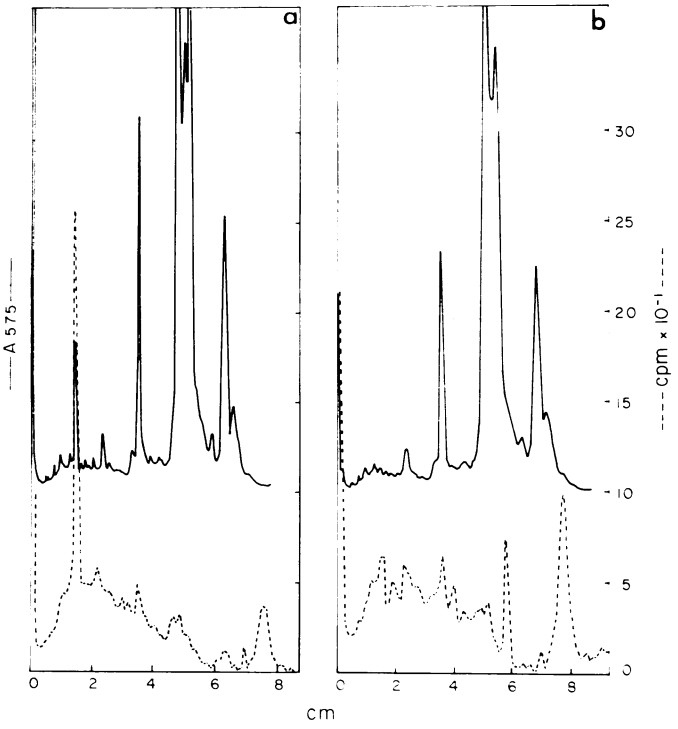

FIG. 4. Protein profiles and phosphorylation patterns of *Lemna* chromatin proteins labeled *in vivo* with ^{32}Pi in the presence or absence of abscisic acid. Plants were labeled for 8 days on medium containing 4 μCi/ml ^{32}Pi and 1 mM KH_2PO_4. Abscisic acid (final concentration 10 μg ABA/ml) was added to one-half of the culture for the last 24 hr. Chromatin was prepared, and the samples representing proteins from equal amounts of DNA were subjected to acid-urea gel electrophoresis at 2 C for 17 hr at 70 v. After staining, the gels were scanned at 575 nm and sliced into 1-mm slices and counted. A_{575} (——); radioactivity (– – –). a: Control; b: ABA-treated.

The results shown in Figure 3 indicate that manipulation of the ionic environment of the nucleus induces a significant alteration in the pattern of *in vitro* protein phosphorylation. Figure 3 shows the effect of including 0.15 M KCl (3a) or

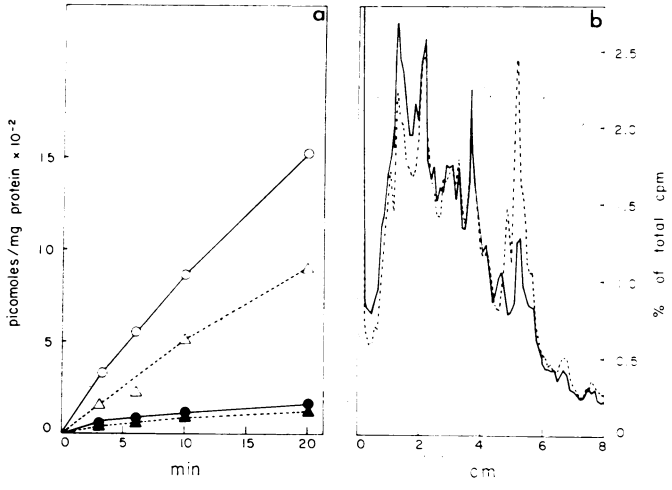

FIG. 5. Effect of abscisic acid on nuclear protein kinase and chromatin phosphorylation patterns labeled *in vitro*. Cultures of *Lemna* were treated for 18 hr in the presence and absence of abscisic acid. Nuclei were isolated from these two cultures and incubated in ^{32}P-ATP for periods of up to 20 min for kinase assay and for 20 min for gel analysis. a: Protein kinase assays of nuclei from control (○●) and ABA-treated tissue (△▲). Kinase activity was determined by assessing the transfer of ^{32}Pi from ^{32}P-ATP to cold acid-insoluble material as described under "Materials and Methods." Incubation of nuclei was carried out in the presence (open symbols) or the absence, (closed symbols) of casein (5 mg/ml). b: Acid-urea gel electrophoretic profiles of phosphorylated chromatin proteins from tissue incubated in the presence or absence of abscisic acid. After labeling the two protein samples were prepared and separated by urea-acetic acid gel electrophoresis, the gels were stained, scanned, and sliced into 1-mm slices. The ordinate represents the percentage of the total activity on the gel in each slice. About 30 μg were loaded on each gel, and the specific radioactivity of the chromatin proteins was 2050 cpm/μg for control and 801 cpm/μg for ABA-treated sample.

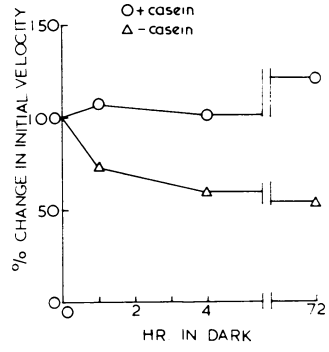

FIG. 6. Effect of placing *Lemna* in darkness on the level of protein kinase associated with isolated nuclei. Cultures of *Lemna* were either placed in darkness for 1, 4, and 72 hr or left under the normal light regime and the nuclear fraction isolated. Nuclear protein kinase activity of the dark-treated and the control cultures were determined in the presence and absence of casein (5 mg/ml) by incubating for 3, 6, 10, and 20 min. Smooth curves were drawn through the data and initial velocities determined in the normal way. The ordinate represents the percentage difference in initial velocity between cultures placed in darkness and control cultures. Kinase assayed in the presence of casein (○); kinase assayed in the absence of casein (△).

0.1 M NaCl (3b) in the incubation medium during labeling of nuclei isolated from 21-hr embryos. The major effect of including both salts in the labeling medium was to increase selectively the phosphorylation of the broad band between 3.5 and 4.5 cm. The effect on the fastest running component, which is acid-soluble, is about 3-fold. The net effect is to produce a phosphorylation profile which is similar but not identical to nuclei from 5-hr-old embryos.

Effect of Abscisic Acid and Light/Dark Transitions on Chromatin Protein Phosphorylation in *Lemna*. Figure 4 shows phosphorylation patterns of *Lemna* chromatin proteins grown for 8 days on ^{32}Pi medium with or without the addition of abscisic acid 24 hr before harvesting. During this time the culture increased 30- to 50-fold and thus the intracellular phosphates assume a specific activity identical with that of the medium. We have calculated the amount of Pi attached to the chromatin proteins by summing the radioactivity on the acrylamide gels (Fig. 4) and determining the radioactivity of the DNA from which these chromatin proteins were separated. The determined ratios of inorganic phosphate in DNA to chromatin protein were from control 103 and ABA 89. These figures are not significantly different. The level of Pi attached to protein is comparable to that found in animal systems (3). There are several dramatic differences in the phosphorylation profile after ABA treatment. There is an almost total loss of a slowly migrating band at 1.5 cm and an increase in two bands running at 6 and 8 cm.

Figure 5 shows an attempt to duplicate this response by isolating nuclei from control and ABA-treated *Lemna* and phosphorylating *in vitro* with labeled ATP. Kinase assays of these two batches of nuclei are shown in Figure 5a. With ABA treatment there is about a 50% reduction in the phosphorylation rate in both the presence or absence of added casein. The phosphorylation profiles are shown in Figure 5b. It can be seen that the dramatic decline in the band at 1.5 cm is now hardly detectable but there is some increase in the band at 6 cm. This change can thus be produced both *in vivo* and *in vitro*.

One of the major events in the life of any green plant is the diurnal variation, the light/dark cycle. We have studied the effect on the endogenous nuclear kinase which results from placing *Lemna* in the dark (Fig. 6). When assayed in the presence of casein the nuclear-associated protein kinase showed slight increases in activity up to 72 hr in darkness. In contrast the rate of endogenous phosphorylation fell quickly in darkness and continued up to a 50% lower rate for 72 hr. Acrylamide gel analysis of the *in vitro* protein phosphorylation profiles of control cultures and those in darkness for 72 hr were also carried out but no significant qualitative differences occur as a result of the dark treatment and thus the results are not shown here.

DISCUSSION

This paper provides evidence for changes in chromatin protein phosphorylation patterns during barley germination and after ABA treatment of *Lemna*. The changes in germination do not appear to be the result of tissue differentiation since profiles of root and shoot phosphorylated chromatin proteins appear to be very similar. The abscisic acid effects on *Lemna* phosphorylation (Fig. 4), while equally dramatic, appeared to be confined to only three bands and although they can be partly duplicated *in vitro* their significance must await further study.

The level of protein phosphorylation measured in the experiment shown in Figure 4 is comparable to that found in animal systems (3). It can be calculated, using the chromatin analysis shown in our previous paper (7) that the acidic protein fraction has 0.1% by weight of phosphorus. As shown by Kleinsmith *et al.* (3) this yields a value of 1 serine phosphate/25 amino acid residues which, for a phosphorylated protein of mol wt 40,000 (7), is about 8 Pi/molecule.

Perhaps the most interesting result is the selective effect of low concentrations of K and NaCl on protein phosphorylation patterns in barley nuclei (Fig. 3). The ionic strength is known to be an important determinant of chromatin structure (1). Furthermore, the nuclear kinase shows a lack of substrate specificity as evidenced by its ready ability to phosphorylate casein (Figs. 1, 5, and 6), phosvitin, and calf thymus histone (Keates and Trewavas, unpublished data) and endogenous nuclear proteins. This must be contrasted with the degree of specificity of nuclear protein phosphorylation shown in Figures 2, 3, 5, and 6 of the previous paper (7) and Figures 1 to 5 in this paper which show that only certain chromatin proteins become phosphorylated. Consideration of these three facts suggests that the structure of the chromatin imposes a specificity on phosphorylation by exposing only a limited number of available protein serines to an essentially nonspecific kinase. If this is true, it further suggests that the alterations in phosphorylation patterns, shown, *e.g.*, in Figure 1, may merely be a *post factum* expression of some other alteration in chromatin structure rather than an implicit alteration of kinase activity.

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