PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Phosphorylation of Nuclear Proteins

R. A. Laskey

Phil. Trans. R. Soc. Lond. B 1983 **302**, 143-150
doi: 10.1098/rstb.1983.0047

Phil. Trans. R. Soc. Lond. B **302**, 143–150 (1983) [143]
Printed in Great Britain

Phosphorylation of nuclear proteins

By R. A. Laskey
M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

Many nuclear proteins are phosphorylated: they range from enzymes to several structural proteins such as histones, non-histone chromosomal proteins and the nuclear lamins. The pattern of phosphorylation varies through the cell cycle. Although histone H1 is phosphorylated during interphase its phosphorylation increases sharply during mitosis. Histone H3, chromosomal protein HMG 14 and lamins A, B and C all show reversible phosphorylation during mitosis. Several nuclear kinases have been characterized, including one that increases during mitosis and phosphorylates H1 *in vitro*.

Factors have been demonstrated in maturing amphibian oocytes and mitotic mammalian cells that induce chromosome condensation and breakdown of the nuclear membrane. The possibility that they are autocatalytic protein kinases is considered. The location of histone phosphorylation sites within the nucleosome is consistent with a role for phosphorylation in modulating chromatin folding.

Introduction

Protein phosphorylation is widely used for biological regulation (reviewed by Cohen (1982)) and the nucleus is a major site of regulatory interactions in the cell. Nevertheless there is relatively little information on regulation by protein phosphorylation within the nucleus. There are, however, widespread observations on the phosphorylation of nuclear proteins, changes in patterns of phosphorylation and their correlation with cellular events. This paper will survey observations on phosphorylation of nuclear proteins, with particular emphasis on the structural proteins of the nucleus and changes observed in their phosphorylation during the cell division cycle. For a more detailed discussion of the phosphorylation of nuclear enzymes and its effect on enzyme activity see the paper by Rose *et al.* (this symposium).

In the first section of this paper the wide range of phosphorylated nuclear proteins will be illustrated. Then changes in phosphorylation during the mitotic cycle will be considered, followed by an account of some of the nuclear kinase activities reported so far. The next section will describe factors that appear cyclically in mitotic and meiotic cells and induce nuclear breakdown and chromosome condensation. The possibility that they are autocatalytic protein kinases will be considered. The final section will discuss the possible roles of phosphorylation of nuclear structural proteins and will consider the possibility that protein phosphorylation is generally used to modulate the structure of various components of the nucleus.

Protein phosphorylation in the interphase nucleus

The pattern of protein phosphorylation in the nucleus is complex. Table 1 selects examples to illustrate the range. For example, all of the histones have been reported to be phosphorylated in at least some circumstances, yet only histone H2A is maintained at a clear constant level of phosphorylation whereas phosphorylation of H2B is rare and phosphorylation of H3 and H1

Table 1. Examples of nuclear proteins that are phosphorylated during interphase

	selected references
histone H1 histone H2A histone H4	references in Hochhauser *et al.* (1981)
poly(A) polymerase	Rose & Jacob (1980)
RNA polymerase I	Duceman *et al.* (1981); Dahmus (1981*b*)
RNA polymerase II	Dahmus (1976)
SV40 T antigen	Baumann & Hand (1982)
nuclear protein kinases	Dahmus (1981*a*); Rose *et al.* (1981)

is cyclical (for references see Gurley *et al.* (1978*a*, *b*), Paulson & Taylor (1982) and Hochhauser *et al.* (1981)).

The four major non-histone chromosomal proteins (referred to as the 'high-mobility group' or HMG proteins) show characteristic levels of phosphorylation. The two larger proteins, HMG1 and HMG2, which appear to be correlated with chromosome replication, are generally not phosphorylated, whereas the two smaller proteins, HMG14 and HMG17, which appear to be correlated with transcriptionally active chromatin, are phosphorylated (Saffer & Glazer 1982). Furthermore, there are clear changes in phosphorylation of HMG14 during the cell cycle (see below).

Phosphorylation of many other non-histone nuclear proteins has been observed (reviewed by Hochhauser *et al.* (1981)). Most of these are unidentified so their significance is unclear, but it has frequently been speculated that their phosphorylation is involved in transcriptional activation (reviewed by Hochhauser *et al.* (1981)). This hypothesis is difficult to test for uncharacterized proteins, but much clearer examples of regulation of nuclear activities come from studies of phosphorylation of eukaryotic RNA polymerases I and II (Duceman *et al.* 1981; Dahmus 1976, 1981*b*) and poly(A) polymerase (Rose & Jacob 1980). In each case phosphorylation by a nuclear protein kinase increases enzyme activity.

Changes in phosphorylation of nuclear proteins during the mitotic cycle

The most striking variations in phosphorylation of nuclear proteins occur during the mitotic cycle. As summarized in table 2, cyclical changes have been observed for histones, a non-histone chromosomal protein of the HMG group and the proteins of the nuclear lamina, a structure that lies just inside the nuclear membrane. In addition, in *Xenopus* oocytes a major protein of the nucleoplasm becomes very highly phosphorylated at the time of nuclear breakdown during meiosis.

Table 2. Nuclear proteins that become phosphorylated during mitosis or meiosis

protein	location in nucleus	references
histone H1	nucleosome	references in Paulson & Taylor (1982)
histone H3	nucleosome core	
HMG 14	active nucleosomes	
lamins A, B, and C	nuclear lamina	Gerace & Blobel (1980)
nucleoplasmin	nucleoplasm	S. Taylor & R. Laskey, unpublished

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The most extensively studied of these proteins are the histones H3 and H1. Of these H3 shows a much simpler pattern of phosphorylation, being selectively phosphorylated at serine residue 10 during mitosis but apparently remaining unphosphorylated for the remainder of the cell cycle (Paulson & Taylor 1982). H1 also shows increased phosphorylation during mitosis, with phosphorylation occurring at up to six sites on both the amino-terminal and the carboxy-terminal domains at serine and threonine residues (Gurley *et al.* 1978*a* (and references therein); Paulson & Taylor 1982 (and references therein); Lennox *et al.* 1982). Unlike H3, however, H1 also shows phosphorylation during interphase (figure 1). Its pattern of interphase phosphorylation is complex and complicated by the existence of several H1 subtypes, which may show independently varied phosphorylation patterns. For example, in mouse teratocarcinoma F9 cells, five different H1 subtypes give rise to at least ten phosphorylated derivatives, resulting in at least 15 modification variants of H1 in this one cell type (Lennox *et al.* 1982). An equally complex picture emerges from a comparison of phosphorylated H1 subtypes in different Chinese hamster cell lines (Wilkinson *et al.* 1982). A detailed study of phosphorylation of H1 at different sites in interphase in HeLa cells has revealed reciprocal patterns of phosphorylation at

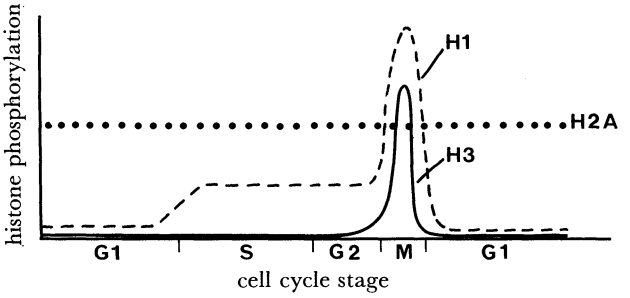

FIGURE 1. Patterns of histone phosphorylation during the cell cycle of CHO cells. The figure shows a simplification of the data of Gurley *et al.* (1978*a*, *b*). See text for details of the complexity of phosphorylation of H1 during interphase.

two different sites. Phosphorylation at one of these two sites is independent of DNA synthesis, whereas phosphorylation at the other site increases during the S phase and is blocked by inhibition of DNA synthesis (Ajiro *et al.* 1981). This suggests that phosphorylation at this site is characteristic of replicated chromatin and it would be interesting to determine if this plays a role in distinguishing replicated DNA from unreplicated DNA to ensure that each region of the chromosome is replicated only once in any cell cycle (see Laskey *et al.* (1981) for a further discussion of this mechanism).

The occurrence of phosphorylation of H3 and 'superphosphorylation' of H1 during mitosis has led to suggestions that they may either trigger mitosis or cause chromosome condensation. A causal correlation of this sort is supported by the existence of a temperature-sensitive cell line that fails to phosphorylate H3 or H1 at the end of the G2 phase and which also fails to condense its chromosomes (Matsumoto *et al.* 1980; Yasuda *et al.* 1981). Furthermore Bradbury *et al.* (1974) showed that mitosis in *Physarum* could be advanced by 40 min by the addition of a relatively crude preparation of a nuclear protein kinase that phosphorylated H1 *in vitro*. The experimental design of seeking biological activity of proteins added to the culture medium of a slime mould is bizarre, yet its validity has been confirmed by the clear demonstration that similar administration of exogenous histone H3 to *Physarum* results in preferential incorporation of the histone into chromatin (Prior *et al.* 1980). Nevertheless the extent to which histone

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

phosphorylation itself is causal is uncertain. Thus Gurley *et al.* (1978*a*) pointed out that phosphorylation does not precede chromosome condensation in the manner expected of a trigger, but both processes show similar kinetics. Similarly, Krystal & Poccia (1981) have shown that phosphorylation can occur without condensation, thus arguing that, although it may be necessary for condensation, it is not sufficient. An informative comparison has been made in *Tetrahymena* by Allis & Gorovsky (1981), who found that increased phosphorylation of H1 and H3 occurs during division of micronuclei of *Tetrahymena* even though nuclear breakdown does not occur. This excludes the trivial explanation that enhanced phosphorylation is caused by exposure to a cytoplasmic kinase.

Thus evidence of a causal role for histone phosphorylation in mitosis is suggestive but circumstantial. Perhaps the observations that other major nuclear proteins are also selectively phosphorylated during mitosis may help to clarify the relationship. HMG 14 and the proteins of the nuclear lamina, lamins A, B and C, are also phosphorylated during mitosis (Paulson & Taylor 1982; Gerace & Blobel 1980). The isoelectric point of the lamins changes substantially at the time of nuclear membrane breakdown, consistent with addition of multiple phosphate residues. The location of the lamina makes its phosphorylation an attractive candidate for a role in nuclear membrane breakdown during mitosis.

During meiosis of amphibian oocytes another major change in nuclear protein phosphorylation is observed. Maller *et al.* (1977) reported a twofold to threefold increase in ^{32}P phosphate incorporation into total nuclear protein. Fractionation reveals that much of the increased phosphate incorporation is into one nuclear protein, nucleoplasmin (S. Taylor & R. A. Laskey, unpublished observation). This protein undergoes a concomitant change in isoelectric point of at least 0.5 pH at the time of nuclear breakdown. It can then be resolved into at least 30 electrophoretic variants (C. Dingwall & R. A. Laskey, unpublished). Because it is the most abundant protein of the oocyte's nucleoplasm, occurring at 5–8 mg ml^{-1} in the nucleus (Laskey & Earnshaw 1980), this level of phosphorylation represents an increase in nuclear phosphate content of 6000 times greater than the total phosphate content of the oocyte's chromosomal DNA. This could obviously have profound effects on the structure of the other components of the nucleus. Because Krohne & Franke (1980) have argued that nucleoplasmin is widespread in vertebrate somatic nuclei, it is an interesting candidate for a role in the mitotic cycle.

Protein kinases from the nucleus

There have been numerous reports of protein kinases from eukaryotic nuclei. Kish & Kleinsmith (1974) reported at least 12 protein kinase activities from bovine liver nuclei. Several protein kinases have been purified from nuclei, though it is not possible to assess the extent to which different reports refer to the same kinase.

The most thoroughly characterized nuclear protein kinases are not stimulated by cyclic nucleotides nor inhibited by the regulatory subunit or heat-stable inhibitor of the cyclic-AMP-dependent protein kinase. Thornburg & Lindell (1977) and Thornburg *et al.* (1978) purified two enzymes, NI and NII. NI displays the substrate preferences:

casein > phosvitin > histone H2B.

It has only one polypeptide chain of 25 kDa apparently occurring as a dimer. NII has the substrate preferences:

phosvitin > casein > histone H2B,

and appears to be an $\alpha\alpha'\beta_2$ tetramer. Kinases closely resembling NII have been purified by Dahmus (1981*a*) and by Rose *et al.* (1981), who have shown stimulation of RNA polymerase I. Both groups observed autophosphorylation of the kinase subunit of approximately 25 kDa. Both groups have also purified enzymes sharing some properties with NI of Thornburg & Lindell (1977). Dahmus (1976) observed stimulation of RNA polymerase II by a nuclear protein kinase and Rose & Jacob (1980) observed stimulation of poly(A) polymerase. Nuclear protein kinases with different properties have been purified (Baydoun *et al.* 1981).

In terms of relevance to the cell cycle perhaps the most interesting nuclear protein kinase is that described and partly purified by Lake (1973). This kinase appears in late G2 phase and remains active through mitosis. It phosphorylates H1 at seven specific sites and it would be interesting to know whether it corresponds to any of the purified nuclear kinases and whether it can also phosphorylate the other nuclear proteins that become phosphorylated during mitosis, particularly the nuclear lamins.

Cyclical appearance of factors that induce mitosis and meiosis

Several types of study show that the behaviour of nuclear proteins can be modulated by factors from the cytoplasm. Thus chromosome condensation and nuclear membrane breakdown can be triggered by fusing a mitotic cell to a cell in S phase (Johnson & Rao 1970) or by

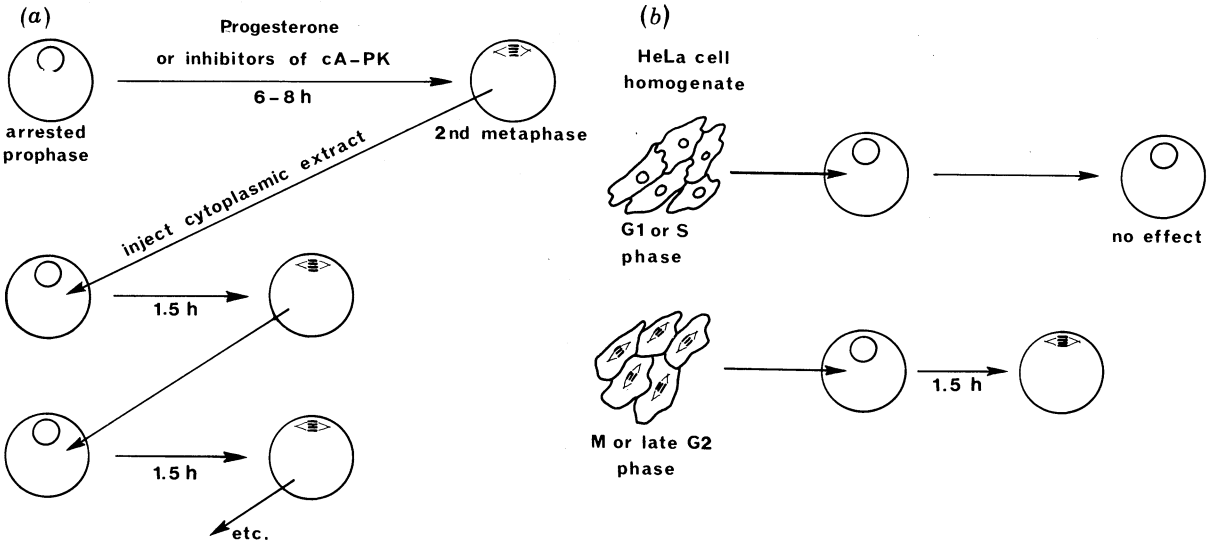

Figure 2. (*a*) The production and amplification of maturation-promoting factor from *Xenopus* oocytes (for references see Wu & Gerhart 1980); (*b*) the cyclical appearance of maturation-promoting activity from HeLa cells demonstrated by injection into *Xenopus* oocytes (Adlakha *et al.* 1982).

injecting an interphase nucleus into a maturing *Xenopus* oocyte (Gurdon 1970). The factors responsible have not been identified but in both cases active fractions can be prepared that will induce premature meiotic nuclear breakdown in injected recipient *Xenopus* oocytes. As shown in figure 2, this activity (called maturation-promoting factor, or MPF) can be derived from maturing amphibian oocytes or from mitotic HeLa cells. In both cases activity is destroyed by exposure to phosphatases or calcium ions and is stabilized by phosphatase inhibitors, suggesting that the active factor is phosphorylated (Wu & Gerhart 1980; Adlakha *et al.* 1982). Furthermore

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the purest active preparations so far have retained a protein kinase (Wu & Gerhart 1980). A striking feature of this maturation-promoting activity is its autoamplification (figure 2*a*). Thus it arises approximately 6 h after progesterone administration (or inhibition of the cyclic-AMP-dependent protein kinase that appears to mediate the progesterone response (Maller & Krebs 1977)). However, when an extract containing maturation-promoting factor is injected into recipient oocytes, meiotic nuclear breakdown is much faster, taking only approximately 1.5 h and bypassing the cyclic-AMP-dependent kinase and a cycloheximide-sensitive step. Therefore a maturation-promoting factor arises during mitosis or meiosis and has properties that would be expected of an autocatalytic kinase. It would be interesting to know whether it is indeed a protein kinase and if so what is its substrate specificity. In particular it would be interesting to know whether it resembles the kinase that Lake (1973) observed only during mitosis, and which shows specificity for histone H1.

A dynamic role for protein phosphorylation in changing the structure of nuclear components

The phosphorylation of several structural proteins of the nucleus is clearly correlated with structural changes. Histones H3 and H1, HMG 14 and the lamins are all reversibly phosphorylated during mitosis. This suggests that phosphorylation may be used to alter the structure and interaction of nuclear components. Figure 3 illustrates the current model for the structure of the nucleosome (Klug *et al.* 1980). The importance of histones H1 and H3 for structural

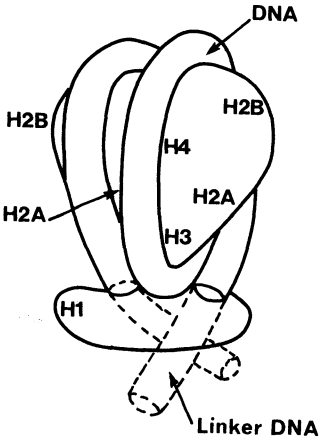

Figure 3. A model for the structure of the nucleosome showing the approximate areas occupied by histones H3 and H1. Note that the exact position and shape of histone H1 and the precise path of DNA in this region are not known. The second molecules of histones H3 and H4 are not shown because they would occupy sites on the concealed surface of the particle. Redrawn with modifications from Klug *et al.* (1980).

variations is clear. Both occupy sites of contact with DNA where it enters and leaves the nucleosome. Their phosphorylation could exert a maximum effect on the interaction of the nucleosome with the DNA of the internucleosomal linker consistent with a role in the higher-order packing of nucleosomes during chromosome condensation. Conversely they are in a position that could influence nucleosome assembly profoundly, and a correlation has been observed between the assembly of nucleosomes at the physiological spacing of 200 base pairs

and the phosphorylation of proteins that closely resemble H3 and H1 (W. C. Earnshaw, F. Mechali & R. A. Laskey, in prep.).

A causal role for protein phosphorylation in changing the structure of nuclear components during the cell division cycle remains to be demonstrated directly. Taken together, however, the observations of cyclical phosphorylation and cyclical appearance of factors promoting premature chromosome condensation and nuclear membrane breakdown emphasize the need for further studies of nuclear protein phosphorylation.

I am grateful to Francisca Mechali and Barbara Rodbard for help in preparing the manuscript.

References

Adlakha, R. C., Sahasrabuddhe, C. G., Wright, D. A., Lindsey, W. F. & Rao, P. N. 1982 Localization of mitotic factors on metaphase chromosomes. *J. Cell Sci.* **54**, 193–206.

Ajiro, K., Borun, T. W., Shulman, S. D., McFadden, G. M. & Cohen, L. H. 1981 Comparison of the structures of human histones 1A and 1B and their intramolecular phosphorylation sites during the HeLa S-3 cell cycle. *Biochemistry, Wash.* **20**, 1454–1464.

Allis, C. D. & Gorovsky, M. A. 1981 Histone phosphorylation in macro- and micronuclei of tetrahymena thermophila. *Biochemistry, Wash.* **20**, 3828–3833.

Baumann, E. A. & Hand, R. 1982 Phosphorylation and dephosphorylation alter the structure of D2 hybrid T antigen. *J. Virol.* **44**, 78–87.

Baydoun, H., Hoppe, J. & Wagner, K. G. 1981 Quaternary structure and ligand specificity of two messenger-independent protein kinases from porcine liver nuclei. In *Cold Spring Harbor Conference on Cell Proliferation* (ed. O. M. Rosen & E. G. Krebs), bk B, vols A and B, pp. 1095–1125. New York: Cold Spring Harbor Laboratory.

Bradbury, E. M., Inglis, R. J., Matthews, H. R. & Langan, T. A. 1974 Molecular basis of control of mitotic cell division in eukaryotes. *Nature, Lond.* **249**, 553–556.

Cohen, P. 1982 The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature, Lond.* **296**, 613–620.

Dahmus, M. E. 1976 Stimulation of ascites tumor RNA polymerase II by protein kinase. *Biochemistry, Wash.* **15**, 1821–1829.

Dahmus, M. E. 1981*a* Purification and properties of calf thymus casein kinases I and II. *J. biol. Chem.* **256**, 3319–3325.

Dahmus, M. E. 1981*b* Calf thymus RNA polymerases I and II do not contain subunits structurally related to casein kinases I and II. *J. biol. Chem.* **256**, 11 239–11 243.

Duceman, B. W., Rose, K. M. & Jacob, S. T. 1981 Activation of purified hepatoma RNA polymerase I by homologous protein kinase NII. *J. biol. Chem.* **256**, 10 755–10 758.

Gerace, L. & Blobel, G. 1980 The nuclear envelope lamina is reversibly depolymerised during mitosis. *Cell* **19**, 277–287.

Gurdon, J. B. 1970 Nuclear transplantation and the control of gene activity in animal development. *Proc. R. Soc. Lond.* B **176**, 303–314.

Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L. & Tobey, R. A. 1978*a* Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur. J. Biochem.* **84**, 1–15.

Gurley, L. R., Walter, R. A. & Tobey, R. A. 1978*b* Sequential phosphorylation of histone subfractions in the Chinese hamster cell cycle. *J. biol. Chem.* **250**, 3936–3944.

Hochhauser, S. J., Stein, J. L. & Stein, G. S. 1981 Gene expression and cell cycle regulation. *Int. Rev. Cytol.* **71**, 95–243.

Johnson, R. T. & Rao, P. N. 1970 Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. *Nature, Lond.* **226**, 717–722.

Kish, V. M. & Kleinsmith, L. J. 1974 Nuclear protein kinases. *J. biol. Chem.* **249**, 750–760.

Klug, A., Rhodes, D., Smith, J., Finch, J. T. & Thomas, J. O. 1980 A low resolution model for histone core of the nucleosome. *Nature, Lond.* **287**, 509–516.

Krohne, G. & Franke, W. W. 1980 A major soluble acidic protein located in nuclei of diverse vertebrate species. *Expl Cell Res.* **129**, 167–189.

Krystal, G. W. & Poccia, D. L. 1981 Phosphorylation of cleavage stage histone H1 in mitotic and prematurely condensed chromosomes. *Expl Cell Res.* **134**, 41–48.

Lake, R. S. 1973 Further characterization of the F1-histone phosphokinase of metaphase-arrested animal cells. *J. Cell Biol.* **58**, 317–331.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Laskey, R. A. & Earnshaw, W. C. 1980 Nucleosome assembly. *Nature, Lond.* **286**, 763–767.
Laskey, R. A., Harland, R. M., Earnshaw, W. C. & Dingwall, C. 1981 Chromatin assembly and the co-ordination of DNA replication in the eukaryotic chromosome. In *International Cell Biology* (ed. H. Schweiger), pp. 163–167. Berlin: Springer-Verlag.
Lennox, R. W., Oshima, R. G. & Cohen, L. H. 1982 The H1 histones and their interphase phosphorylated states in differentiated and undifferentiated cell lines derived from murine teratocarcinomas. *J. biol. Chem.* **257**, 5183–5189.
Maller, J., Wu, M. & Gerhart, J. C. 1977 Changes in protein phosphorylation accompanying maturation of *Xenopus laevis* oocytes. *Devl Biol.* **58**, 295–312.
Maller, J. L. & Krebs, E. G. 1977 Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. *J. biol. Chem.* **252**, 1712–1718.
Matsumoto, Y., Yasuda, H., Mita, S., Marunouchi, T. & Yamada, M. 1980 Evidence for the involvement of H1 histone phosphorylation in chromosome condensation. *Nature, Lond.* **284**, 181–183.
Paulson, J. R. & Taylor, S. S. 1982 Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. *J. biol. Chem.* **257**, 6064–6072.
Prior, C. P., Cantor, C. R., Johnson, E. M. & Allfrey, V. G. 1980 Incorporation of exogenous pyrene-labeled histone into *Physarum* chromatin: a system for studying changes in nucleosomes assembled *in vivo*. *Cell* **20**, 597–608.
Rose, K. M. & Jacob, S. T. 1980 Phosphorylation of nuclear poly(adenylic acid) polymerase by protein kinase: mechanism of enhanced poly(adenylic acid) synthesis. *Biochemistry, Wash.* **19**, 1472–1476.
Rose, K. M., Bell, L. E., Siefken, D. A. & Jacob, S. T. 1981 A heparin-sensitive nuclear protein kinase. *J. biol. Chem.* **256**, 7468–7477.
Saffer, J. D. & Glazer, R. I. 1982 The phosphorylation of high mobility group proteins 14 and 17 and their distribution in chromatin. *J. biol. Chem.* **257**, 4655–4660.
Thornburg, W. & Lindell, T. J. 1977 Purification of rat liver nuclear protein kinase NII. *J. biol. Chem.* **252**, 6660–6665.
Thornburg, W., O'Malley, A. F. & Lindell, T. J. 1978 Purification of rat liver nuclear protein kinase NI. *J. biol. Chem.* **253**, 4638–4641.
Wilkinson, D. J., Shinde, B. G. & Hohmann, P. 1982 Cell-specific phosphorylation of H1 histone subtypes among different Chinese hamster cell lines in interphase. *J. biol. Chem.* **257**, 1247–1252.
Wu, M. & Gerhart, J. C. 1980 Partial purification and characterization of the maturation-promoting factor from eggs of *Xenopus laevis*. *Devl Biol.* **79**, 463–477.
Yasuda, H., Matsumoto, Y., Mita, S., Marunouchi, T. & Yamada, M. 1981 A mouse temperature-sensitive mutant defective in H1 histone phosphorylation is defective in deoxyribonucleic acid svnthesis and chromosome condensation. *Biochemistry, Wash.* **20**, 4414–4419.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES