Histone Phosphorylation in Macro- and Micronuclei of *Tetrahymena thermophila*†

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ABSTRACT: The patterns of histone phosphorylation in amitotically dividing, transcriptionally active macronuclei and in mitotically dividing, transcriptionally inert micronuclei of the ciliated protozoan *Tetrahymena thermophila* have been analyzed. Taken together, the major phosphorylation events in these two nuclei and their dependence on cell growth and/or division are remarkably similar to those in mammalian cells. Phosphorylation of H1-type proteins occurs in both nuclei and is positively correlated with growth and/or division. Phosphorylation of histone H3 is also positively correlated with growth and/or division but occurs only in micronuclei. Phosphorylation of histone H2A is relatively independent of growth state and occurs largely, if not exclusively, in macronuclei. Given the unique partition of nuclear functions between macro- and micronuclei, these results, coupled with previously reported temporal correlations between specific histone phoshporylations and cell cycle events in mammalian cells [Gurley, L. R., Tobey, R. A., Walters, R. A., Hildebrand, C. E., Hohmann, P. G., D'Anna, J. A., Barham, S. S., & Deaven, L. L. (1978a) in *Cell Cycle Regulation* (Jeter, J. R., Cameron, J. L., Padilla, G. M., & Zimmerman, A. M., Eds.) pp 37–60, Academic Press, New York], allow insights into the functions of histone phosphorylations. Specifically, a nonmitotic function for extensive H1 phosphorylation and a unique mitotic function for H3 phosphorylation are clearly indicated. A new role for H2A phosphorylation in the regulation of transcriptional activity is also proposed.

Specific postsynthetic modifications of histones may be correlated with particular aspects of chromatin structure and function (Allfrey, 1971; Smulson, 1979; Isenberg, 1979; Allis et al., 1980a). Several possible functions, not necessarily mutually exclusive, have been ascribed to various histone phosphorylation events. These include possible roles in histone transport and/or deposition, in heterochromatinization, in mitotic chromosome condensation, in interphase chromatin structure, and in genetic activity [see Gurley et al. (1978a), Isenberg (1979), and the Discussion of this paper for details].

Macro- and micronuclei of the ciliated protozoan *Tetrahymena thermophila* offer a unique opportunity to explore possible relationships between histone phosphorylation and chromatin structure and function. In vegetatively growing cells, both macro- and micronuclei replicate and divide (on average) once per cell cycle. Micronuclei divide mitotically but are transcriptionally inert. Macronuclei divide amitotically but are transcriptionally active [see Gorovsky (1973) and Gorovsky et al. (1978) for a summary of the properties of macro- and micronuclei]. Histone phosphorylation events that are associated with mitosis should, therefore, be unique to micronuclei, while those associated with genetic activity should be restricted to macronuclei. Phosphorylation events occurring in interphase but unrelated to genetic activity (e.g., associated with DNA replication) and those common to both mitotic and amitotic nuclear division (e.g., chromatid separation) should occur in both nuclei.

In this report, we utilize high-resolution, two-dimensional polyacrylamide gel electrophoresis coupled with in vivo incorporation of ^{32}P to characterize the phosphorylation patterns of macro- and micronuclear histones of vegetatively growing and starved *Tetrahymena*.

Experimental Procedures

Cell Culture and Isolation of Nuclei. *Tetrahymena thermophila*, strain B-VII, were used in all of the studies reported here. For studies involving growing cells, cells were cultured axenically in enriched proteose peptone as described previously (Gorovsky et al., 1975). Cells were harvested at densities not exceeding 200 000 cells/mL. For studies involving starved cells, growing cells (200 000 cells/mL) were pelleted, washed 1 time with 10 mM Tris, pH 7.4, and resuspended in an equal volume of Tris. Cells were starved in Tris for at least 12 h. Macro- and micronuclei were isolated from growing and starved cells according to published procedures (Gorovsky et al., 1975).

[^{32}P]*Phosphate Labeling Conditions.* Cells were labeled during growth by adding [^{32}P]orthophosphate (16 μCi/mL) directly to the medium either from the start of the culture (continuous label) or during the last cell doubling (2–3 h) prior to the isolation of nuclei. No differences were observed in the results obtained by either labeling procedure.

Extraction of Histones. Histones were extracted as previously described (Allis et al., 1979), utilizing precautions to avoid artifactual protein losses.

Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was performed as described previously (Allis et al., 1979, 1980a,b), using either short (11 cm) Triton–acid–urea or long (30 cm) acid–urea first-dimension gels. After being stained with Coomassie blue and destained (Allis et al., 1979), the second-dimension gels were photographed and directly dried for autoradiography (with ^{32}P-labeled proteins). ^{14}C-Labeled ink was used to mark the borders of dried gels, allowing accurate superimposition of the staining profiles with the autoradiograph. Photographs were then taken of autoradiographs, and these were printed to match those of the corresponding staining profiles. In this way, it was possible to determine precisely which proteins were labeled. In some cases, gels were stained with fast green and scanned at 630 nm in a Gilford spectrophotometer (Gorovsky et al., 1970). Isotopic counting of stained proteins from one- or two-di-

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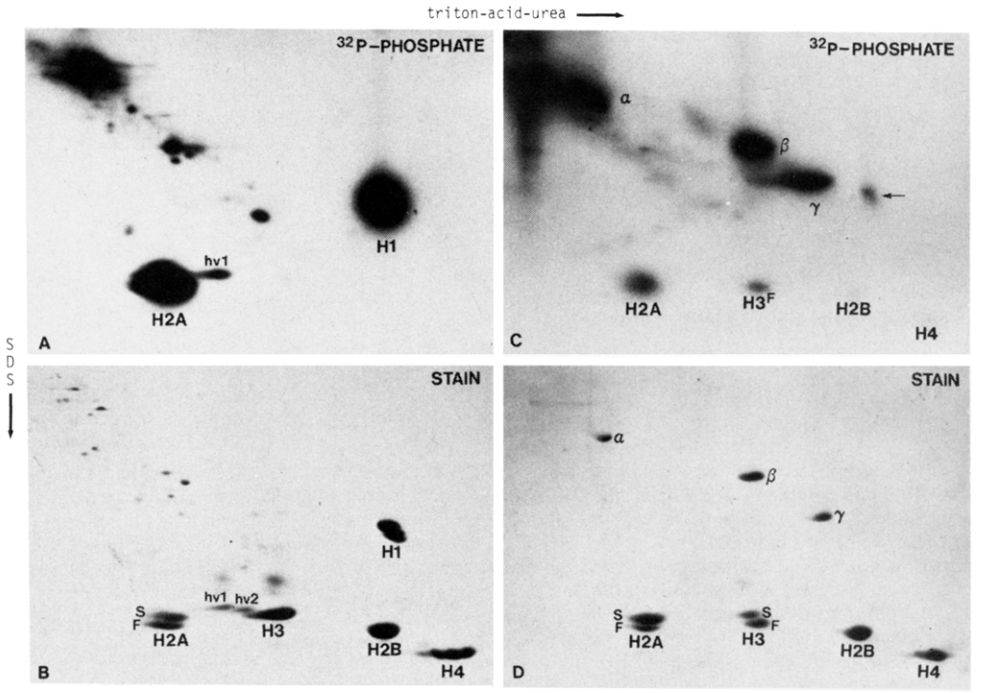

FIGURE 1: Patterns of phosphorylation of macro- and micronuclear histones from growing cells. Two-dimensional gels (Triton–acid–urea by $NaDodSO_4$) of acid-soluble proteins from macro- (A and B) and micronuclei (C and D) are shown. These nuclei were isolated from cells grown continuously in the presence of [^{32}P]phosphate. Besides the major histones H1, H2A, H2B, H3, and H4, two relatively minor macronuclear-specific histone variants, hv1 and hv2 [see Allis et al. (1980b)], are resolved in the staining profile of acid-soluble material from macronuclei (B). Its corresponding autoradiograph of ^{32}P-labeled proteins is shown in (A). Besides histones H2A, H2B, and H4, both forms of micronuclear H3, $H3^S$ and $H3^F$ [see Allis et al. (1979, 1980a)], and the micronuclear-specific, linker DNA associated proteins, α, β, and γ (Allis et al., 1979), are revealed in the staining profile of acid-soluble material from micronuclei (D). The corresponding autoradiograph (C) shows ^{32}P-labeled micronuclear proteins. A small amount of macronuclear contamination in this micronuclear preparation is indicated by the presence of some phosphate in the area of macronuclear H1 in (C) (arrow). The autoradiographs in (A) and (C) were exposed for identical lengths of time.

mensional gels was as previously described (Allis et al., 1980b).

Results

Macro- and Micronuclear Histones. A brief description of the histone complement of *Tetrahymena* macro- and micronuclei is required to understand their phosphorylation patterns. Macronuclei contain five typical histone fractions: H1, H2A, H2B, H3, and H4 (Figure 1B). Of these major fractions, only H2A contains primary sequence variants ($H2A^F$ and $H2A^S$) which are easily separated on polyacrylamide gels containing sodium dodecyl sulfate ($NaDodSO_4$). Macronuclei also contain two quantitatively minor fractions which can be resolved from each other and from the other histones in Triton–acid–urea gels. One of these, hv2, has been clearly identified as a variant of H3. The other variant, hv1, is similar in some ways to H2A, but its peptide map differs from that of both $H2A^F$ and $H2A^S$, making its identity less certain (Allis et al., 1980b).

Micronuclei do not contain H1 of the type found in macronuclei (Gorovsky & Keevert, 1975; Johmann & Gorovsky, 1976a). Instead, they contain three peptides (labeled α, β, and γ in Figure 1D) which, like H1 in other nuclei, appear to be localized in the linker region of micronuclear chromatin (Allis et al., 1979). Although not yet characterized in detail, these three peptides are assumed to be "H1-like" in function. The major forms of histones H2A, H2B, and H4 in micronuclei are similar to, if not identical with, their macronuclear counterparts. However, micronuclear H3 exists in two electrophoretically distinct forms (Allis et al., 1979). $H3^S$ (the slower migrating form) is indistinguishable from macronuclear H3. $H3^F$ is unique to micronuclei and has been shown to be derived from $H3^S$ by a physiologically regulated proteolytic cleavage which removes six residues from the amino terminus of $H3^S$ (Allis et al., 1980a). Finally, micronuclei do not contain the minor histone variants, hv1 and hv2 (Allis et al., 1980b).

Histone Phosphorylation in Macro- and Micronuclei of Growing Cells. Figure 1 shows a two-dimensional electrophoretic separation (Triton–acid–urea by $NaDodSO_4$) of acid-soluble proteins extracted from macro- (A and B) and micronuclei (C and D) isolated from log-phase cells (cell density = 200 000 cells/mL) grown continuously in [^{32}P]-orthophosphate. When the pattern of stained macronuclear proteins (Figure 1B) is compared to the corresponding autoradiograph (Figure 1A) of the histones, only H2A, H1, and hv1 are significantly phosphorylated in macronuclei [also see Allis et al. (1980b)]. In micronuclei (Figure 1C,D), the three H1-like proteins, α, β, and γ, are highly phosphorylated. Most strikingly, $H3^F$, the micronucleus-specific proteolytically processed form of H3, is also labeled. Micronuclear $H3^S$, which is the precursor of $H3^F$, is not phosphorylated. Since similar amounts of macro- and micronuclear protein were analyzed in Figure 1 (compare the staining intensities of Figure 1 B,D), and the autoradiographs (A and C) were exposed for identical times, the failure to detect ^{32}P incorporation into macronuclear H3 suggests that H3 phosphorylation in *Tetrahymena* is micronuclear specific. Small amounts of ^{32}P are also usually detected over H2B and H4 in micronuclei, but not in macronuclei.

For α, β, γ, and $H3^F$ and micronuclear H2B and H4, we can conclude that ^{32}P incorporation is indeed micronuclear specific and not due to macronuclear contamination, since macronuclear forms of these proteins either do not exist (α, β, γ, and $H3^F$) or are unphosphorylated (H2B and H4). The situation with micronuclear H2A is slightly less clear. Although some ^{32}P is typically observed with micronuclear H2A

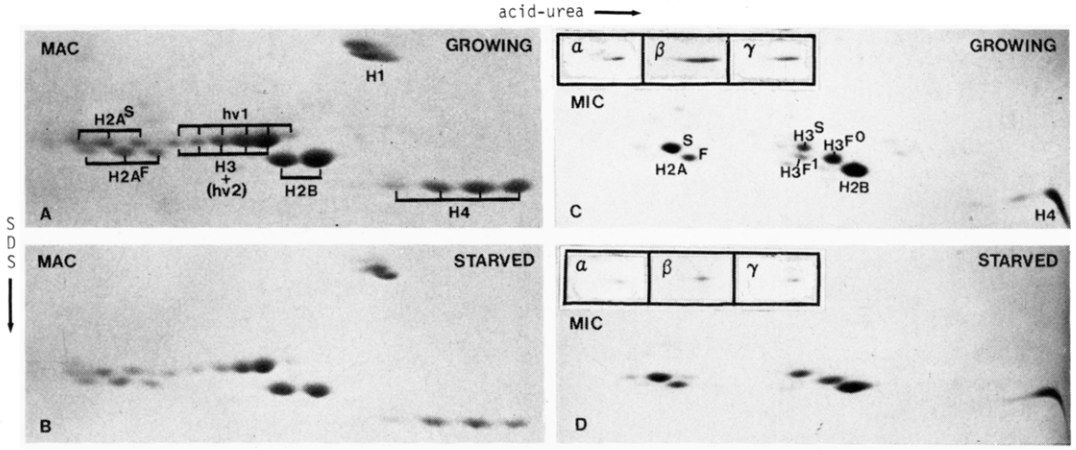

FIGURE 2: Electrophoretic heterogeneity of macro- and micronuclear histones from growing and starved cells. Two-dimensional gels (long, acid–urea by $NaDodSO_4$) of acid-soluble proteins from macro- (A and B) and micronuclei (C and D) of growing (A and C) and starved (B and D) cells are shown. No attempt has been made to label all of the modified subspecies from each of the macronuclear histones in (A). Instead, brackets mark modified and unmodified subspecies within each histone class. For a description of the positions of hv1 and hv2 in these acid-urea gels, see Allis et al. (1980b). Because α and β migrate so much slower than the other micronuclear histones in long acid–urea gels, it is impossible (given the dimension of our second-dimension gel plates) to include all micronuclear histones in one second-dimension gel. For that reason, α, β, and γ are shown in the inserts given in (C) and (D). γ in (C) was taken from a different gel (of micronuclei from growing cells) because it electrophoresed poorly in the gel illustrated in (C).

Table I: Comparison of ^{32}P Incorporation into Macro- and Micronuclear H2A during Growth

	^{32}P cpm		
histone[a]	mac	mic	mic/mac
H2A	26 623	1587	0.06
H1	18 630	518[b]	0.03

[a] Comparable amounts of ^{32}P-labeled histone (labeled continuously during growth) were fractioned on two-dimensional gels (Triton–acid–urea by $NaDodSO_4$). Stained spots corresponding to macronuclear H2A and H1, micronuclear H2A, and macronuclear H1 cross-contaminating the micronuclear preparation were excised and counted. [b] ^{32}P counts here must result from macronuclear contamination within the micronuclear preparation since micronuclei do not contain any macronuclear type H1 (Gorovsky & Keevert, 1975; Johmann & Gorovsky, 1976a).

(Figure 1C), it is always much less than that observed with macronuclear H2A (Figure 1A). In fact, it is likely that most, if not all, of this radioactivity is due to macronuclear contamination. Previous estimates of macronuclear contamination in micronuclear preparations range from 5 to 15% (Gorovsky et al., 1975). In one experiment, the H2A regions of gels like those shown in Figure 1 were cut out and counted by scintillation spectrometry (Table I). Micronuclear H2A had approximately 6% the radioactive phosphate as similar amounts of macronuclear H2A. Since in situ immunofluorescence indicates that micronuclei do not contain macronuclear type H1 (Johmann & Gorovsky, 1976a), the presence of small amounts of ^{32}P incorporation at the position of macronuclear H1 in Figure 1C (arrow) indicates low levels of macronuclear contamination in this micronuclear preparation. In the same gels from which the H2A regions were excised and counted, ^{32}P incorporation into the macronuclear H1 region of the micronuclear gel was 3% of that in H1 on the macronuclear gel (Table I). Thus, at least half of the low levels of ^{32}P incorporated into micronuclear H2A is almost certainly due to macronuclear contamination. This is likely to be a minimal estimate of macronuclear contamination since it is often difficult to recover small amounts of macronuclear H1 (Allis et al., 1979). We conclude, therefore, that micronuclear H2A incorporates little (approximately 30-fold less than comparable amounts of macronuclear H2A, Table I) or no radioactive phosphate.

Phosphorylation-Associated Electrophoretic Heterogeneity of Macro- and Micronuclear Histones. Histones which are modified by phosphorylation (or acetylation) migrate more slowly in acid–urea gels, owing to their decreased net positive charge. Careful alignments of the stain patterns and the autoradiographs of the gels shown in Figure 1 show that the ^{32}P radioactivity migrated slightly slower in the first dimension than the stainable proteins. However, the gel system used for the first dimension in Figure 1 (short, 11 cm, Triton–acid–urea gels), while necessary to separate hv1 and hv2 in macronuclei, does not completely resolve modified histone subspecies. Better resolution of modified subspecies can be obtained by using long (30 cm) acid–urea gels in the first dimension. Figure 2 shows such a high-resolution, two-dimensional gel separation (long, acid–urea by $NaDodSO_4$) of macro- (A) and micronuclear (C) histones isolated from growing cells. As previously described (Gorovsky et al., 1978; Johmann & Gorovsky, 1976b), extensive heterogeneity is observed with each of the macronuclear histones. Autoradiographic analysis (data not shown) of ^{32}P-labeled macronuclear histones again shows phosphate to be associated with H1, H2A, and hv1 (hv1 comigrates with H3 and its subspecies in acid–urea gels without Triton; Allis et al., 1980b). Fluorographs of macronuclear histones labeled in vivo with sodium [^3H]acetate show that acetate is associated with the modified subspecies of all of the macronuclear histones except H1 (Allis et al., 1980b; C. D. Allis and M. A. Gorovsky, unpublished experiments). Thus, H1 is the only macronuclear histone for which the relative amounts of slower migrating subspecies can be used unambiguously to assess the level of phosphorylation; either the others are unphosphorylated (H2B, H3, H4, and hv2) or subspecies are either acetylated and/or phosphorylated (H2A and hv1). It is interesting to note that the phosphorylated subspecies of macronuclear H1 migrate more slowly in both the first (acid–urea) and second ($NaDodSO_4$) dimensions. This has also been observed with the H1 for *Drosophila* (Billings et al., 1979).

Figure 2C shows a typical separation of acid-soluble micronuclear proteins in this gel system. Some heterogeneity is seen with almost all the micronuclear histones. For H2A, $H3^S$, H2B, and H4, this heterogeneity is variable and is probably due largely to macronuclear contamination. Thus, growth-related changes in the phosphorylation of micronuclear

Table II: Comparison of [^{32}P]Phosphate Incorporation into Macronuclear Histones during Growth and Starvation[a]

histone	sp act.[b]		
	growing	starved	growing/starved
H2A	5842	5512	1.06
hv1[c]	431	443	0.97
H1[d]	7001	3036	2.31

[a] Cells were labeled for one generation (100–200 000 cells/mL) with [^{32}P]phosphate and divided into two equal aliquots. Macronuclei were isolated immediately from one aliquot; the remaining cells were starved for 24 h before their macronuclei were isolated. Histones were analyzed on short acid–urea gels (Glover et al., 1981). [b] ^{32}P cpm/integrated absorbance of fast green stain at 630 nm in arbitrary units (Gorovsky et al., 1970). [c] hv1 comigrates with H3 on short acid–urea gels; macronuclear H3 is not phosphorylated (see Figure 1A). [d] H1 comigrates with H2B on short acid–urea gels; macronuclear H2B is not phosphorylated (see Figure 1A).

H2A, H2B, H3^S, and H4 cannot easily be assessed electrophoretically. These complications do not hold for α, β, γ, and H3^F, since these proteins are unique to micronuclei. In growing cells, we have consistently observed (Figure 2C) that H3^F consists of two subspecies, a faster migrating (in the first dimension) unmodified form, H3$^{F^0}$, and a slower, phosphorylated form, H3$^{F^1}$. Autoradiographic analysis of ^{32}P-labeled micronuclear histones resolved on gels such as that shown in Figure 2C shows that H3$^{F^1}$ is indeed phosphorylated while H3$^{F^0}$ is not (data not shown). α, β, and γ typically are resolved into long trailing tails (Figure 2C) which also label with ^{32}P. Analyses of micronuclear histone labeled in vivo with sodium [^{3}H]acetate have not revealed any acetate associated with α, β, γ, or H3^F. These observations suggest that the heterogeneity observed with α, β, and H3^F in this gel system is due solely to phosphorylation.

Comparison of Histone Phosphorylation in Growing and Starved Cells. From the above discussion, we conclude that relative amounts of slower migrating modified subspecies can be used to assess the level of phosphorylation associated with macronuclear H1 and micronuclear α, β, γ, and H3^F. We have used this criterion to evaluate whether phosphorylation of any of these histones is growth related. Figure 2 shows two-dimensional electrophoretic separations (long, acid–urea by NaDodSO$_4$) of macro- (A and B) and micronuclear (C and D) histones isolated from growing (A and C) and nongrowing (starved, B and D) cells. In macronuclei, only H1 differs significantly in the two physiological states: The proportion of slower migrating subspecies is significantly greater in the H1 from growing cells. Thus, as described previously (Gorovsky et al., 1974; Glover et al., 1981), macronuclear H1 is phosphorylated in a growth-dependent fashion. The absence of detectable differences in the electrophoretic heterogeneity of H2A and hv1 suggests they are not phosphorylated in a growth-dependent fashion. However, this interpretation is complicated somewhat by the fact that these histones are also acetylated. For determination of whether H2A or hv1 displays any growth/division related changes in phosphorylation, cells were grown for one generation on [^{32}P]orthophosphate and either harvested immediately or starved for 24 h in a phosphate-free medium (10 mM Tris). Macronuclear histones were isolated and electrophoresed on one-dimensional acid–urea gels. The H2A, hv1, and H1 regions were quantitated by densitometry, excised, and counted (see Experimental Procedures). The specific activity (^{32}P/unit of stain) of H1 was 2.3 times higher in growing than in starved cells (Table II), in good agreement with the expected difference in phosphorylation between these two growth states (Glover et al., 1981). In contrast, the phosphorylation levels of H2A and hv1 were essentially the same in growing and starved cells. Thus, as in mammalian cells, phosphorylation of H2A (and of the H2A-like variant, hv1) varies little, if at all, in different growth states.

When the micronuclear histones from growing (Figure 2C) and starved (Figure 2D) cells are compared, it is clear that a greater fraction of α, β, γ, and H3^F exists as slower migrating subspecies (more phosphorylated) in growing cells. Thus, we conclude that the phosphorylation of these micronuclear histones is growth and/or division related.

Discussion

Some discussion of the nature of the phosphorylated subspecies being studied seems warranted. The phosphorylation events described above cannot result from acid-labile phosphoryaltion, since all of our observations were made on acid-extracted histones. Poly(ADP) ribosylation [see Hayaishi & Ueda (1977)] could account for some of the ^{32}P incorporation and for altered mobilities of phosphorylated subspecies. However, in macronuclei, the radioactive phosphate associated with H1, H2A, and hv1 is not removed by treatment with alkali (0.1 N NaOH for 60 min at room temperature). This treatment also does not reduce the electrophoretic heterogeneity associated with these histones. Treatment with alkaline phosphatase, on the other hand, greatly reduces the electrophoretic heterogeneity and the radioactive phosphate associated with these macronuclear histones (C. D. Allis, C. V. C. Glover, and M A. Gorovsky, unpublished experiments). Alkali treatment of micronuclear histones has not been performed. However, phosphatase treatment greatly reduces the electrophoretic heterogeneity of α, β, γ, and H3^F. Moreover, we have recently observed that most of the ^{32}P incorporated into H3^F is associated with a serine residue near the amino terminus (C. V. C. Glover, C. D. Allis, J. Williams, M. A. Gorovsky, and G. N. Abraham, unpublished experiments). On the basis of these observations, we think it likely that most of the events described here represent direct phosphorylations and not poly(ADP) ribosylations. We also note that the criteria we have applied to characterize these phosphorylation events (^{32}P incorporation, electrophoretic heterogeneity, and phosphatase treatment) are those commonly used in studies of cell-cycle-dependent phosphorylation in other systems and that the term "phosphorylation" does not necessarily imply that the precise nature of the phosphate-containing, modified subspecies is known.

Histone phosphorylation has been extensively studied in other biological systems. The most detailed studies concern H1 phosphorylation. Largely on the basis of studies of mammalian cells in culture, Gurley and collaborators [see Gurley et al. (1978a)] have suggested that there may be (at least) two types of growth- and/or division-related H1 phosphorylation, interphase and mitotic. During interphase, many (but not all) H1 molecules in Chinese hamster ovary (CHO) cells are phosphorylated at one or more of the three serines in the carboxy-terminal portion of H1. At mitosis, H1 molecules are phosphorylated not only at the interphase sites but also at additional serines and threonines in both halves of the molecule. Mitotic H1 phosphorylation (frequently referred to as superphosphorylation) involves most, if not all, H1 molecules and results in many H1 molecules having four to six phosphates.

It has been suggested that interphase H1 phosphorylation plays a role in submicroscopic changes in chromatin structure postulated to occur throughout interphase (Gurley et al., 1978a,b). Bradbury and his colleagues (Bradbury et al., 1973,

1974a,b; Inglis et al., 1976) observed high levels of phosphorylation preceding mitosis in the slime mold *Physarum* and suggested that H1 phosphorylation might function as a trigger for mitosis. In a series of detailed studies with synchronized CHO cells in culture, Gurley and his collaborators (Gurley et al., 1978b) concluded that an increase in H1 phosphorylation at mitosis was correlated with the sum of prophase and metaphase cells, while the decrease in mitotic H1 phosphorylation was correlated with the exit of cells from metaphase or anaphase. Most importantly, no mitotic superphosphorylation was found in the earliest preprophase stages of chromatin condensation. These studies led Gurley and his collaborators to conclude that, in CHO cells, mitotic H1 phosphorylation was not a trigger of mitosis (i.e., did not precede the initiation of chromatin condensation) but was precisely correlated with the existence of "the chromosome structural state per se".

Our finding (Gorovsky et al., 1974; this study) that histone H1 is extensively phosphorylated in the amitotically dividing macronucleus of growing/dividing cells also argues that H1 phosphorylation does not act solely as a mitotic trigger or exclusively to promote chromosome condensation during mitosis. It should also be noted that in unsynchronized, growing *Tetrahymena* the majority of macronuclear H1 molecules are phosphorylated, and many molecules contain three to five phosphate groups, suggesting that even hyperphosphorylated H1 molecules are not uniquely associated with mitotic events. Also, we have recently observed (Glover et al., 1981) that macronuclear H1 can be hyperphosphorylated in the absence of growth and division when starved cells are subjected to stress (e.g., heat shock). These studies of macronuclear H1 argue strongly that H1 phosphorylation plays a role in some process(es) completely unrelated to mitosis.

The three micronuclear-specific H1 analogues, α, β, and γ, are also phosphorylated in a growth- or division-dependent manner. It seems likely that these phosphorylation events are related to mitotic chromosome condensation. However, it is also possible that phosphorylation of H1 and H1-like molecules in both macro- and micronuclei could be due to the involvement of H1 phosphorylation in some process common to mitotic and amitotic division. Unfortunately, methods are not currently available to synchronize the nuclear events in mass cultures of *Tetrahymena thermophila* to determine the timing of specific phosphorylation events in the cell cycle.

Phosphorylation of histone H3 has also been observed during mitosis in several types of mammalian cells in culture [see Gurley et al. (1978a) for a review]. In CHO cells, H3 phosphorylation and dephosphorylation parallel those of mitotic H1, again suggesting a relationship with the presence of mitotic chromosomes. Whether mitotic H1 and H3 phosphorylations are causes or simply consequences of mitotic chromosome condensation in CHO cells is not clear (Gurley et al., 1978a).

The hypothesis that phosphorylation of H3 plays a specific role in mitosis is strongly supported by our studies. We have observed that $H3^F$, the micronuclear-specific, proteolytically processed form of H3, is phosphorylated in growing and dividing cells. Neither macronuclear H3 nor the micronuclear precursor of $H3^F$ ($H3^S$) appears to be phosphorylated. This spatial localization of H3 phosphorylation within a mitotic nucleus, coupled with its absence in an amitotic nucleus in the same cell, fulfills precisely the expectations based on the hypothesis of a mitosis-specific function for H3 phosphorylation which emerged from temporal correlations observed in mammalian cells. It should also be noted that mitosis in the micronucleus occurs without breakdown of the nuclear envelope [see Flickinger (1965)]. Thus, mitosis-specific phosphorylation of H3 (or of any other micronuclear histone) cannot be due to trivial exposure to a cytoplasmic kinase.

Large-scale phosphorylation of histone H2A has been found to occur throughout the cell cycle in mammalian cells [see Gurley et al. (1978a)]. It has been suggested that the amount of H2A phosphorylation is positively correlated with the content of constitutive heterochromatin in cell lines derived from different species of deer mice (Gurley et al., 1978b; Halleck & Gurley, 1980). We have found that macronuclear H2A is extensively phosphorylated to similar extents in growing and starved cells. Thus, as in mammalian cells, macronuclear H2A phosphorylation appears to be constitutive. Since there are no easily detectable satellite DNAs and few, if any, highly repeated sequences in macronuclear DNA (Yao & Gorovsky, 1974), it seems unlikely that H2A phosphorylation is related simply to the presence of constitutive heterochromatin. This conclusion is supported by our finding that in micronuclei H2A incorporates little or no phosphate during growth. To our knowledge, this is the only reported case of a dividing nucleus in which H2A is not phosphorylated. Since micronuclei are germinal nuclei and, in fact, contain some repeated DNA sequences not found in macronuclei (Yao & Gorovsky, 1974), they might be expected to contain even more constitutive heterochromatin than macronuclei. Based on our observation that extensive H2A phosphorylation is macronucleus specific, it seems more likely that this particular phosphorylation event is related to mechanisms which regulate transcription in active nuclei.

We have also observed low levels of ^{32}P incorporation into micronuclear H2B and H4, but not into their macronuclear counterparts. However, the phosphorylation levels of these molecules in micronuclei are considerably less than those discussed above and approach the limits of detection by utilizing our experimental procedures. The significance of these phosphorylations is unclear.

In summary, the major histone phosphorylation events in *Tetrahymena* involve H1-type histones, H2A and H3, and vary with growth state just as they do in mammalian cells, suggesting that the functions of histone phosphorylations, like the histones themselves, are remarkably conserved. The unique partitioning of specific functions between macronuclei (amitotic, transcriptionally active) and micronuclei (mitotic, transcriptionally inert) allows new insights into these functions. Specifically, a nonmitotic function for extensive H1 phosphorylation and a unique mitotic function for H3 phosphorylation are clearly indicated. A new role for H2A phosphorylation in the regulation of transcriptional activity is also proposed.

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Salt Dependence of the Kinetics of the *lac* Repressor–Operator Interaction: Role of Nonoperator Deoxyribonucleic Acid in the Association Reaction†

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ABSTRACT: The kinetics of binding of *lac* repressor protein and operator deoxyribonucleic acid (DNA) have been studied as a function of monovalent and divalent cation concentration. The salt dependence of the association and dissociation rate constants has been interpreted in light of recent theoretical analyses based on Manning's counterion condensation model. The bell-shaped dependence of the association rate constant on salt concentration evidences a role for nonoperator DNA binding in the repressor's search for the operator site on a large DNA molecule. At intermediate mono- or divalent cation concentrations, the association rate goes through a maximum. At lower cation concentrations, it decreases and becomes dependent on DNA concentration; the high affinity of repressor for nonoperator DNA confines the protein to the DNA. At higher cation concentrations, the association rate decreases and becomes dependent on the weak affinity of repressor for nonoperator DNA. The kinetic data are fit to the theory of Berg and Blomberg [Berg, O. G., & Blomberg, C. (1978) *Biophys. Chem. 8*, 271] for the salt dependence of association kinetics with coupled diffusion, using published values of the affinity for nonoperator DNA. From this fit, one-dimensional diffusion of repressor along the DNA chain is estimated to be about 4 times faster on MgDNA than on NaDNA. At higher cation concentrations, the salt dependence of the association and dissociation rate constants is consistent with a preequilibrium mechanism for the association reaction [Lohman, T. M., deHaseth, P. L., & Record, M. T., Jr. (1978) *Biophys. Chem. 8*, 281]. Agreement between literature values (corrected for the presence of Mg^{2+}) and experimental values of the rate constants in the presence of monovalent salt is quite good.

The *lac* repressor protein and its operator site on deoxyribonucleic acid (DNA), plus inducing sugars, comprise the negative control system of the *lac* operon in *Escherichia coli* [for a comprehensive review, see Miller & Reznikoff (1978)]. The interaction of repressor protein and operator DNA has been extensively studied in vitro by the membrane filter technique. The results are consistent with a simple bimolecular reaction of repressor (R) and operator (O)

$$R + O \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RO \qquad (1)$$

where k_a is the association rate constant and k_d is the dissociation rate constant, and where the observed equilibrium association constant, K_{obsd}, is

$$K_{obsd} = \frac{[RO]}{[R][O]} = \frac{k_a}{k_d} \qquad (2)$$

The repressor binds tightly to the operator. In standard buffer, K_{obsd} is about 1×10^{13} M^{-1}, k_a is about 7×10^9 M^{-1} s^{-1}, and k_d is about 6×10^{-4} s^{-1} (Riggs et al., 1970a,b). The bimolecular association of repressor and operator is very rapid, the value of the rate constant k_a being about 2 orders of magnitude greater than expected for a diffusion-limited reaction. The binding parameters are quite sensitive to ionic conditions. For example, a 10-fold increase in ionic strength (from 0.02 to 0.20 M) results in about a 60-fold decrease in the association rate

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