FURTHER CHARACTERIZATION OF THE F1-HISTONE PHOSPHOKINASE OF METAPHASE-ARRESTED ANIMAL CELLS

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ABSTRACT

Exponentially growing Chinese hamster cells are found to contain two major phosphokinase activities with specificity for the phosphorylation of F1 (lysine-rich) histone. These two activities, designated KI and KII, were extracted with 0.35 M NaCl and fractionated in 0.2 M NaCl by Sephadex G-200 gel filtration. KI, which is similar to the ubiquitous cyclic 3′,5′-adenosine monophosphate (cAMP)-dependent phosphokinase, differs from KII by several criteria. KII is mol wt 90,000, cAMP independent, rapidly turned over in vivo, low K_m for ATP, and phosphorylates F1 histone at several unique sites. Comparative examination of metaphase-arrested (M) and counterpart interphase (I) cells for these two activities reveals that KII is responsible for the overall high activity in M-arrested cells. Pulse labeling of cells with ^{32}P during traverse of the G_2-M phase of the cell cycle reveals an in vivo tryptic-phosphopeptide pattern in whole unfractionated F1 which is unique to M cells. Seven major phosphopeptides derived by in vitro phosphorylation of F1 with the KII enzyme correspond to these M cell-specific phosphorylation sites observed in vivo. It is suggested that KII activity predominates during the G_2-M transition and that F1 is its natural in vivo substrate.

INTRODUCTION

In cultured animal cells, the incorporation of ^{32}P into F1 (lysine-rich)histone has been reported to occur predominantly during DNA synthesis (1, 2). This phosphorylation occurs both on old preexisting and on newly synthesized F1 after a short post-translational delay (3).

Experimental evidence has also been presented to suggest that the F1 histone of several metaphase-arrested cell types exists in a highly phosphorylated form (4). Corresponding to this augmented phosphorylation is a transiently high cyclic adenosine monophosphate (cAMP)-independent phosphokinase activity having specificity for F1 (5). In cultured Chinese hamster cells, this metaphase (M) cell phosphokinase has a particularly high specific activity in chromatin which decays during long periods of M arrest or when M cells are released into G_1.

Several basic aspects of this observed phosphorylation-dephosphorylation reaction need to be studied before insight into its regulation and biological function is achieved. Foremost is the question of whether the phosphorylation of F1 which occurs at mitosis is mediated through the same phosphokinase which acts on F1 during interphase (I). As an approach to this basic question, a comparative study has been made of F1 phosphokinases partially purified from M and I Chinese hamster

cells. The tryptic phosphopeptides of both metabolically (in vivo) and enzymatically (in vitro) phosphorylated F1 histone have also been compared. The results reveal that F1-phosphorylation sites in ^{32}P-labeled M cells are distinct from those of I cells. This unique M-cell phosphorylation pattern can be duplicated in vitro with a phosphokinase which is found to be maximally active in M cells.

METHODS

Cells and Culture

Chinese hamster cells (V79) were maintained in monolayer culture and grown exponentially at 36.5°C in suspension culture in Spinner modified Eagle's no. 2 medium supplemented with 10% fetal bovine serum. Vinblastine sulfate-arrested mitotic cells (M) were collected by selective detachment of cells growing in roller bottles in tricine-buffered Eagle's no. 2 medium (tricine EM) as previously described (4).

Extraction of 6% TCA Soluble Proteins

All steps were performed at 0°C. Whole cells washed in Tris-buffered saline or isolated cell fractions were resuspended in 10 vol of deionized water and homogenized 20 strokes in a Dounce homogenizer. An equal volume of 12% TCA was added and the mixture intermittently homogenized for 30 min. After centrifugation at 2,000 *g* for 10 min, the supernatant proteins were precipitated at 25% TCA and the precipitate resuspended for 2 min at room temperature in 1 N NaOH containing 5 mM sodium pyrophosphate. After a second 25% TCA precipitation, the proteins were resuspended in water and reprecipitated in 10 vol of acid-acetone (0.5% vol/vol concn HCl) and washed twice in acetone.

Standard F1-Phosphokinase Assay

Phosphokinase (ATP:F1-histone phosphotransferase) activity was assayed in a 250 μl reaction mixture containing 40 mM Tris-HCl (pH 9.0), 4 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.1% Triton X-100, 10–25 μl of enzyme protein, 50 μg F1 histone, and 20 nmol ATP, added in that order. The reaction was stopped after 10 min at 36°C by chilling and adding TCA to 20%. ^{32}P incorporation was determined as previously described (5) and expressed as either ^{32}P cpm or pmol γ-P_i incorporated into 50 μg of F1. A unit of activity is one pmol of γ-phosphate incorporated into 50 μg of F1 histone per 10 min.

One-Dimensional Tryptic-Phosphopeptide Maps

50 μg of [^{32}P]F1 was dissolved in 100 μl of 8 mM $NaHCO_3$, pH 8.0. Digestion was with 1:25 wt/wt trypsin (Sigma Chemical Co., St. Louis, type XI, DCC treated) at 36.5°C for 5–10 h. The reaction mix was dried *in vacuo* and dissolved in 100 μl of 8.8% formic acid. After counting of the ^{32}P activity, the peptides were redried and resuspended in a volume of 8.8% formic acid such that the spotted volume of each sample contained equal ^{32}P activity. Electrophoresis was at 500 V for 2 h at 4°C in 8.8% formic acid (pH 2.0) on cellulose thin-layer sheets (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). Peptides were located with Ninhydrin spray and phosphopeptides by autoradiography with NS-2T medical X-ray film (Eastman Kodak Co.). Developed X-ray film was photographed using Polaroid type 51 high-contrast film (Polaroid Corp., Cambridge, Mass.). In this system, all ^{32}P peptides migrate between the origin and cathode.

N-Bromosuccinimide (NBS) Cleavage of F1

F1 preparations were subjected to NBS oxidative cleavage (bisection at an internal tyrosine residue) in 50% acetic acid as described by Bustin and Cole (6). NBS (Sigma Chemical Co.) was added at an empirically determined weight ratio of 1 part NBS to 5 parts F1 protein. After 1 h at room temperature, a second addition of NBS at the same level was made for 2 h. Cleavage products were recovered by precipitation overnight at −20°C from 10 vol of acid-acetone. Controls were incubated in 50% acetic acid under parallel conditions.

Polyacrylamide Gel Electrophoresis

Short (8 cm) and long (25 cm) 15% acrylamide gels with 6 M urea and 0.9 N acetic acid, pH 2.7, were run according to Panyim and Chalkley (7). Protein samples were reduced in 0.5 M β-mercaptoethanol in 8 M urea before electrophoresis. Urea-acetic acid gels were stained in 0.2% Amido black in 7.5% acetic acid, 10% methanol, and destained by elution. Densitometer tracings were made at 670 nm in a Gilford 2400 equipped with a 2410 linear transport device (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

1% sodium dodecyl sulfate (SDS) gels containing 13% acrylamide were run according to the methods of Fairbanks et al. (8), except that the bisacrylamide:acrylamide ratio was 1:40. SDS gels were stained in 0.1% Coomassie blue in 7.5% acetic

acid, 50% methanol, and destained by elution in 7.5% acetic acid, 10% methanol.

Phosphokinase Extraction and Separation

Chinese hamster cell phosphokinases having specificity for a variety of substrates including F1 histone are quantitatively solubilized in 0.35 M NaCl without loss of activity (5). Whole cells, nuclei, or purified chromatin were homogenized in reticulocyte standard buffer (RSB, 0.01 M Tris-HCl, 0.01 M NaCl, 0.0015 M $MgCl_2$, pH 7.6) diluted 1:1 with distilled water. The homogenate was brought to 0.35 M NaCl and rehomogenized intermittently for 30 min at 0°C. After initial clarification at 2,000 *g*, the homogenate was centrifuged at 30,000 rpm for 90 min in a no. 30 Spinco rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernatant was brought to 33% ammonium sulfate saturation followed after 30 min by centrifugation at 10,000 *g* for 10 min in a Sorvall SS-34 head (Ivan Sorvall, Inc., Newtown, Conn.). The pellet was resuspended at room temperature in 0.01 M Tris-HCl (pH 8.0), 0.014 M β-mercaptoethanol, 0.20 M NaCl. 1 ml containing 5–10 mg of protein was applied to a 1.5 × 90 cm Sephadex G-200-A120 column equilibrated with the same buffer. Fractions of 2 ml were collected at a flow rate of 10 ml/h and 0.1 ml aliquots were assayed in the standard phosphokinase assay with lysine-rich calf thymus histone (Sigma Chemical Co.) as substrate.

Peak fractions from G-200 columns were pooled and concentrated by vacuum dialysis against 0.01 M Tris-HCl (pH 7.6), 10% glycerol, 0.014 M β-mercaptoethanol, and stored frozen at −20°C. Relative recovery of two phosphokinases designated KI and KII is depicted in Table I. In exponentially growing cells, this fractionation yields over 70% of the total activity as KII with a 20-fold purification. KI which elutes in the void volume is relatively more crude.

In Vivo Labeling with ^{32}P

Pulse labeling of Chinese hamster cells traversing the G_2-M portion of their cell cycle was achieved by adding [^{32}P]phosphoric acid to whole exponentially growing cell populations during accumulation of M cells with vinblastine sulfate. Roller bottle monolayer cultures were rinsed once with warm phosphate-free tricine EM before adding 100 ml of phosphate-free tricine EM supplemented with 2.5 mCi ^{32}P, 0.1 μg/ml vinblastine, and 5% dialyzed fetal bovine serum. After 2–3 h incubation, M cells were separated by selective detachment in regular prewarmed tricine EM without serum. I cells were vigorously rinsed to dislodge residual M cells.

Under these labeling conditions, all collected M cells are representative of that portion of G_2 cells progressing into M for 2–3 h. Although there is some heterogeneity in the collected population due to different total elapsed time spent arrested in M and the time to generate a [^{32}P]ATP pool, such labeling results in an M cell [^{32}P]F1 specific activity two- to threefold higher than in either I cells or random (R) cells labeled for an equivalent time.

RESULTS

Phosphokinases of Chinese Hamster Cells

Two classes of F1-phosphokinase activity are separated from 0.35 M NaCl extracts of whole randomly growing cells by G-200 Sephadex gel-filtration. As seen in Fig. 1 A, gel filtration at 0.2 M NaCl is sufficient to prevent the aggregation of these two enzyme activities which apparently occurs at low salt. At very high salt, above 0.5 M some catalytic activity is shifted to the lower molecular weight range. Estimation of molecular weights by comparison with standard globular proteins on these same columns is >250,000 da for

TABLE I

Recovery and Partial Purification of Chinese Hamster Cell F1 Phosphokinase

Preparation	Volume	Total protein	Total activity	Percent	Specific activity	Fold purification
	(*ml*)	(*mg*)	(*U*)		(*U/mg*)	
Homogenate	20	68	320,000	100	4,720	—
Ammonium sulfate precipitate of 0.35 M NaCl extract protein	1.5	9.9	212,000	66.2	21,400	4.5
Pooled KI peak from Sephadex G-200	1.0	4.5	41,200	12.8	9,140	1.9
Pooled KII peak from Sephadex G-200	1.0	2.5	232,000	72.5	92,700	19.7

phosphokinase I (KI) and ~90,000 da for phosphokinase II (KII). Further chromatography of the KII fraction on DEAE-Sephadex and hydroxyapatite as shown in Fig. 2 failed to resolve KII into more than one activity indicating the likelihood that a single gel filtration step is sufficient to separate this phosphokinase from others.

When nuclear and cytoplasmic fractions are

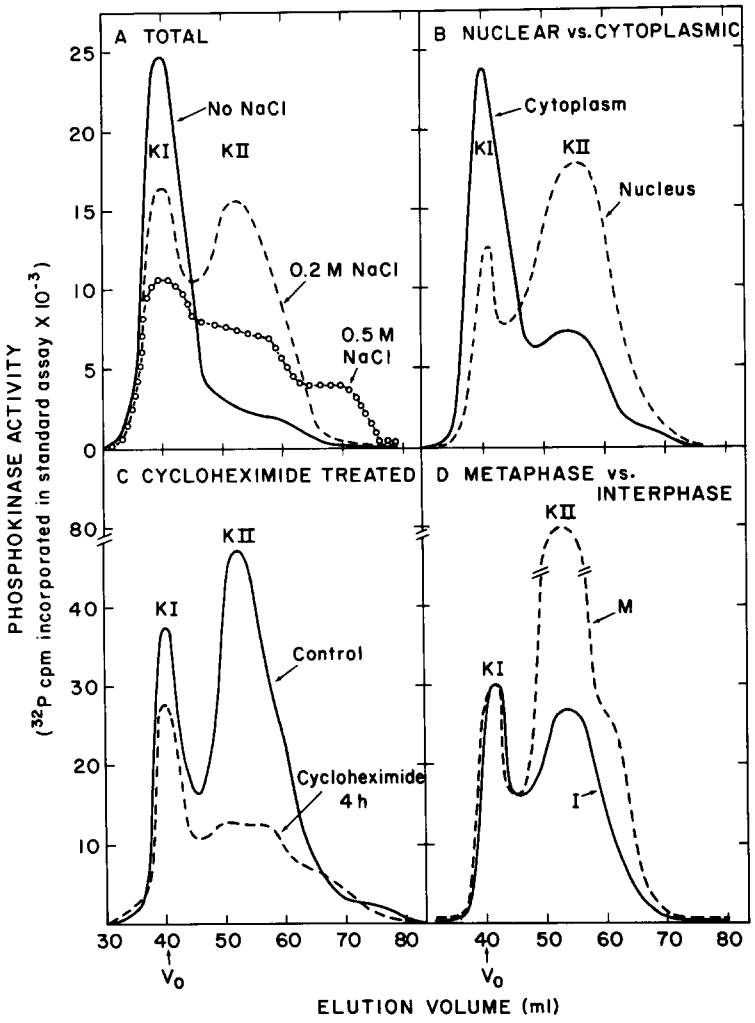

FIGURE 1 Sephadex G-200 elution profiles of phosphokinase activity in 0.35 M NaCl extracts of Chinese hamster cells. Activity was assayed at pH 9.0 with calf thymus lysine-rich histone as substrate. (A) Whole cell phosphokinase extract chromatographed in eluting buffers at various NaCl concentrations. Six aliquots of the same 0.35 M NaCl extract were resuspended after the ammonium sulfate precipitation step (see Methods) in 0.01 M Tris-HCl (pH 8.0), 0.014 M β-mercaptoethanol, and NaCl at 0, 0.1, 0.2, 0.3, 0.4, and 0.5 M. Three profiles are shown. Subsequent samples were chromatographed at 0.2 M NaCl. At this concentration, phosphokinase I (KI) elutes near the excluded volume (V_0) and phosphokinase II (KII) elutes ahead of a bovine serum albumin marker. (B) Relative amounts of KI and KII recovered in nuclear and cytoplasmic fractions separated in hypotonic RSB buffer. (C) Effect of 4 h pretreatment of exponentially growing cells with 3 μg/ml cycloheximide. Total protein recovered in the 0.35 M NaCl extract was less in the cycloheximide sample (5.1 vs. 6.2 mg). Phosphokinase activity has been corrected by multiplying the cycloheximide sample activity by 1.21. (D) Comparison of 4 h M-arrested and I cells. Each 0.35 M NaCl extract from M and I cells of the same starting cell population was adjusted to 7 mg of total protein.

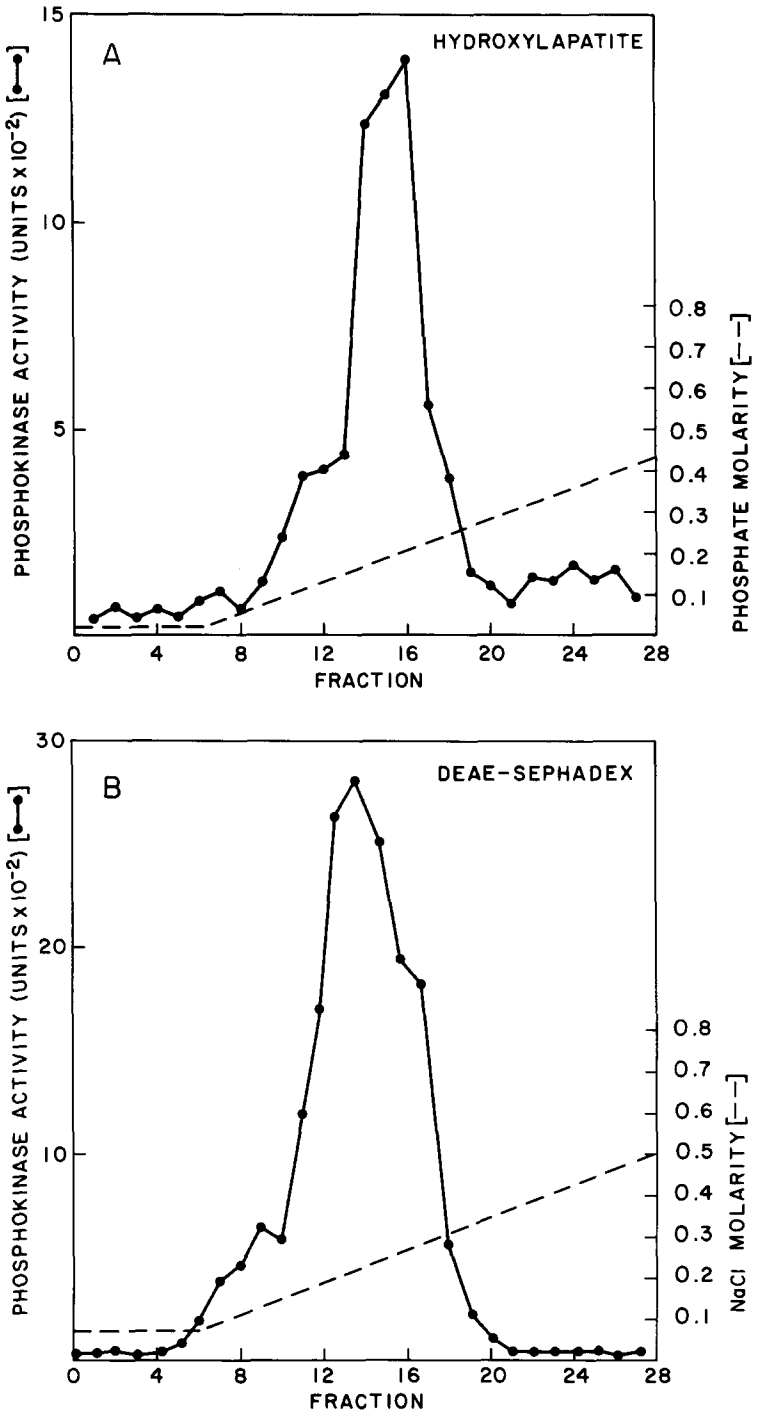

FIGURE 2 DEAE-Sephadex and hydroxylapatite chromatography of KII phosphokinase activity obtained from Sephadex G-200. (A) 2.5 mg of KII was loaded on a 0.9 × 12 cm column of DEAE-Sephadex in 0.01 M Tris-HCl (pH 8.0), 0.014 M β-mercaptoethanol, and eluted with a 50 ml linear gradient of NaCl in the same buffer. Ammonium sulfate precipitated fractions were assayed with calf thymus lysine-rich (F1) histone as substrate. (B) 2.5 mg of KII was loaded on a 0.9 × 12 cm column of hydroxylapatite (5 ml bed volume) in 0.01 M sodium phosphate (pH 6.8), 0.014 M β-mercaptoethanol. Fractions of 2 ml were eluted with a 0.01–0.5 M linear gradient of sodium phosphate at 10°–15°C. Phosphokinase assay was as in (A).

examined for their relative amounts of KI and KII it is found (Fig. 1 B) that both activities are recovered in each cell fraction but in different proportions. Extensive purification of the nuclear fraction by washes in 0.1% Triton X-100 and pelleting through a cushion of 1.7 M sucrose leaves both activities in the nuclear fraction. However, KI is the predominant activity in the cytoplasm and a relatively minor activity in isolated chromatin. Another property of KII as shown in Fig. 1 C is that it is a high turnover activity relative to KI. In the absence of ongoing protein synthesis, KII activity drops rapidly. Since KII is the predominant chromatin associated activity this is consistent with a previous observation on the effect of cycloheximide on chromatin-associated phosphokinase (5) in which total chromatin-associated activity drops to 50% of the original activity within 3 h of cycloheximide treatment.

Lastly, and most important, is a direct comparison of M and I cells, Fig. 1 D. An activity with the gel filtration properties of KII is the one contributing to the high overall F1-phosphokinase activity in M-arrested cells. KI on the other hand is relatively unchanged in activity between the two cell types.

Further, to establish that KI and KII are distinct phosphokinases and not simply interconvertible forms of the same enzyme, the pH optima, response to cAMP, substrate profile, and K_m for ATP have been determined. As seen in Table II, KI differs from KII in that it prefers phosvitin at pH 7.0 and uses protamine as acceptor protein much better than KII at all pH values. KII activities from M and I cells have very similar substrate preferences at all pH values, with a high presence for F1 histone at pH 9.0. KI and KII are similar in that they both phosphorylate F1 very well at pH 9.0. The K_m of KI for ATP with F1 as substrate is 2×10^{-4} M as compared to lower K_m's for KII from either M or I cells (Fig. 3). Slight differences between M and I cell KII are observed, but no significance has yet been ascribed to this in light of the limited degree of purity in these enzyme fractions.

Both phosphokinases exhibit a complex but very reproducible bimodal pH optimum curve as shown in Fig. 4. Neither KI nor KII is stimulated more than 20% by 2×10^{-6} M cAMP at the pH optima for catalytic activity. At low pH, between 6.5 and 8.0, KI is significantly stimulated by cAMP—threefold in this particular experiment. Both, however, exhibit maximal catalytic activity for F1 at pH 9.0. The bimodal nature of the pH curves with F1 acceptor protein is thought to be an effect of pH on the F1 histone rather than the phosphokinases.

Although all the aforementioned properties of KI and KII are being measured with only partially pure enzyme preparations, the measurements serve to show that they are distinct activities, and that M-cell KII, which abounds in M cells, is very similar to I-cell KII.

Proteins in 6% TCA Extracts of Chinese Hamster Cells

Because rigorous purification of F1 and separa-

TABLE II

Substrate Profile for Chinese Hamster Cell Phosphokinase I and II Separated by Sephadex G-200 Gel Filtration

Enzyme source	pH of reaction	Percent of maximum pmole γ-^{32}P incorporated		
		Substrates (50 μg)		
		Histone I	Protamine	Phosvitin
KI interphase cell	7.0	27.4	27.5	100
	8.0	72.0	48.9	50.3
	9.0	86.5	37.4	44.3
KII interphase cell	7.0	44.0	15.7	21.2
	8.0	82.1	18.8	13.1
	9.0	100	25.1	14.4
KII metaphase cell	7.0	43.1	19.2	32.4
	8.0	85.1	10.2	19.3
	9.0	100	10.6	15.4

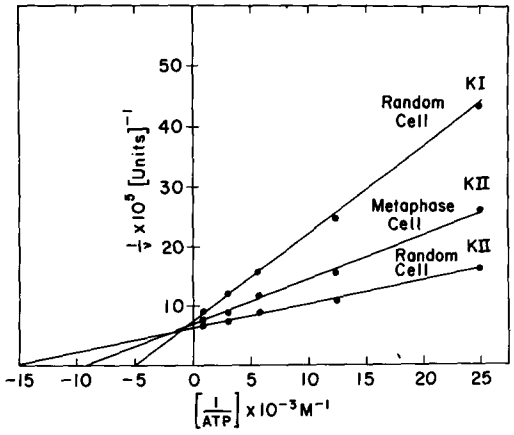

FIGURE 3 Reciprocal plots of ATP vs. velocity (v) for phosphokinase I and II. Partially purified preparations of KI and KII from both R cells and M-arrested cells were adjusted to approximately equal total activity at high ATP. Incubation of the standard reaction mix was for 4 min to give initial reaction rates. Acceptor protein was calf thymus lysine-rich histone (F1) at 100 μg per reaction mix instead of the usual 50 μg to insure saturation. The small difference in apparent K_m of M- and I-cell KII is not reproducible and thus within experimental error.

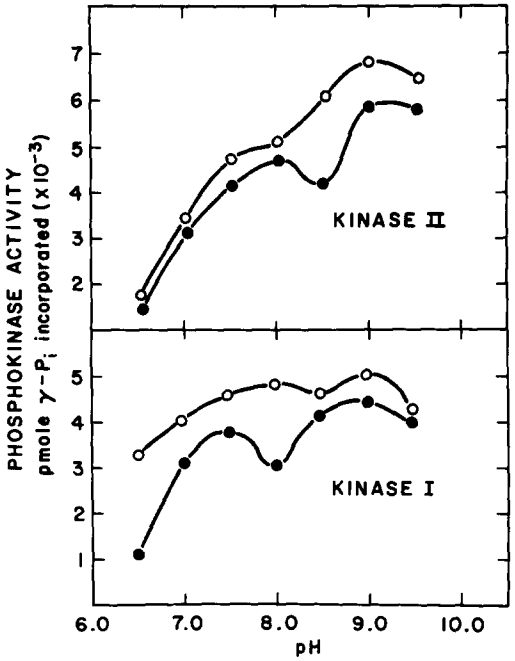

FIGURE 4 pH profile of phosphokinase I and II obtained by G-200 gel filtration of whole exponentially growing Chinese hamster cells. The standard Tris-HCl buffered reaction mix was adjusted to the indicated pH values with HCl. Substrate was calf thymus lysine-rich histone (F1). (●) no cAMP, (○) 2×10^{-6} M cAMP.

tion of its subfractions is impracticable with the small amounts of F1 available from the cultured cells used in these experiments, it was necessary to first characterize the phosphoproteins obtained by a single whole cell TCA extraction of the type used by DeNooij and Westenbrink (9).

Fig. 5 shows a urea-acetic acid gel profile of the proteins in a 6% TCA extract of Chinese hamster cell chromatin. Four major species are identifiable: F1 (with three subcomponents a, b, and c), $F1_o$, P1, and P2. Fig. 6 shows similar preparations run on 13% SDS gels. On SDS gels, where mobility is more independent of charge, P1P2 migrate as a single overlapping band with an apparent mol wt of 15,000. The proteins P1P2, which constitute approximately 4% of the total histones and 16% (on the basis of stain binding) of the TCA-soluble proteins, have the additional properties of being phosphorylated in vivo, containing lysine, and not being cleaved by NBS under conditions which cleave F1. When first observed, these were considered to be either contaminating ribonucleoproteins or proteolytic cleavage products of authentic histones. However, they are present in the same mass ratios with respect to other histones when they are extracted from chromatin, nuclei, or whole cells. Likewise, the fact that P1P2 are present in whole cells extracted with 6% TCA excludes an origin by proteolysis during chromatin isolation. Also, gel *C* in Fig. 6 shows that P1P2 is not a ribonucleoprotein since an equivalent protein is not extracted under parallel conditions from a concentrated microsomal fraction. P1P2 is analogous in all the above properties to a histone-T described by Wigle and Dixon (10).

NBS cleavage products of F1 (denoted N1, the original C-terminal segment and N2, the smaller N-terminal segment [6]) are not separated on urea-acetic acid gels (Fig. 5), but are clearly resolved from F1 and P1P2 on SDS gels. Assignment of molecular weights to F1, N1, and N2 are only approximate, since F1 and presumably its cleavage products exhibits anomolous mobility on SDS gels (11, 12).

Distribution of Phosphorus in the 6% TCA-Soluble Proteins

Examination of the ^{32}P in the TCA-soluble pro-

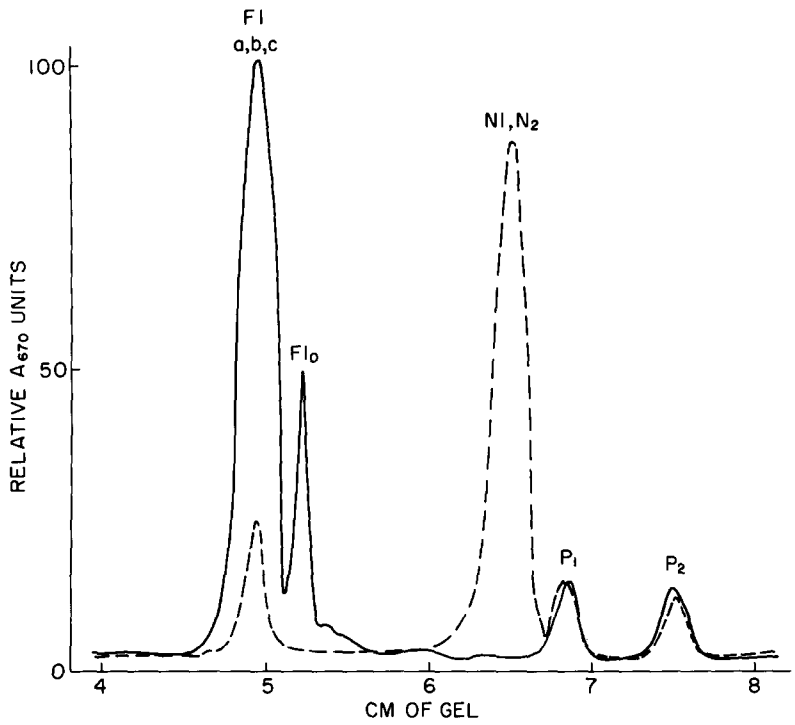

FIGURE 5 Urea-acetic acid polyacrylamide gel electrophoresis of Chinese hamster cell F1-histone preparation before and after cleavage with NBS. In this gel system, 6% TCA-soluble proteins P1 and P2 are separated but the NBS cleavage products are not.

teins was intended to determine whether there is any polarity or meaningful difference in the distribution of phosphorylation sites in F1 of M and I cells.

First, the average distribution of ^{32}P between the two NBS cleavage products was determined in Chinese hamster cells labeled for two generations (24 h) with ^{32}P. This distribution would be expected to represent the average or net steady-state distribution of phosphorus in the two NBS fragments averaged over the entire cell cycle. With such long term labeling, it is found that 26–30% of the radiophosphorus of cleavage products is found in the N2 fragment as shown in Fig. 7. Despite being only about 16% of the total 6% TCA-soluble proteins by mass, P1P2 contains 30% of the total incorporated ^{32}P; indicating that P1P2 is more highly phosphorylated than F1 during long term in vivo labeling.

Second, the distribution of ^{32}P in F1 cleavage products was examined after either in vivo or in vitro phosphorylation. For in vivo labeling, a population of exponentially growing cells was pulse labeled with ^{32}P during a 3 h period of collection of M cells with vinblastine. For in vitro labeling, F1-phosphokinase II from M cells was used. One-half of these phosphorylated F1 preparations was cleaved with NBS as described in Methods and electrophoresed on parallel SDS gels. Results as depicted in Figs. 8 A–C, reveal several facts pertinent to subsequent experiments. Comparison of Figs. 8A and B shows that ^{32}P activity of the M-F1 peak is increased over an equivalent physical amount of I-F1, while there is little difference in the ^{32}P of P1P2 in the same samples. In this respect, P1P2 acts as an internal control to indicate that the difference between in vivo ^{32}P incorporation in M and I-F1 cannot be due to differences in [^{32}P]ATP pool sizes. This conclusion assumes that a common ATP pool is used for all nuclear phosphorylation reactions. As illustrated in Fig. 8 C, P1P2 is phosphorylated only by in vivo labeling; the KII enzyme which is apparently responsible for F1 phosphorylation has virtually no specificity for P1P2 under the standard in vitro reaction conditions. Lastly, and contrary to initial expection, when the ^{32}P activity in cleavage peaks (N1, N2) is analyzed, as was done with the data from Fig. 7, little difference in the ratio of ^{32}P in N1 to that in N2 for M-F1 and I-F1 is found (not tabulated). In

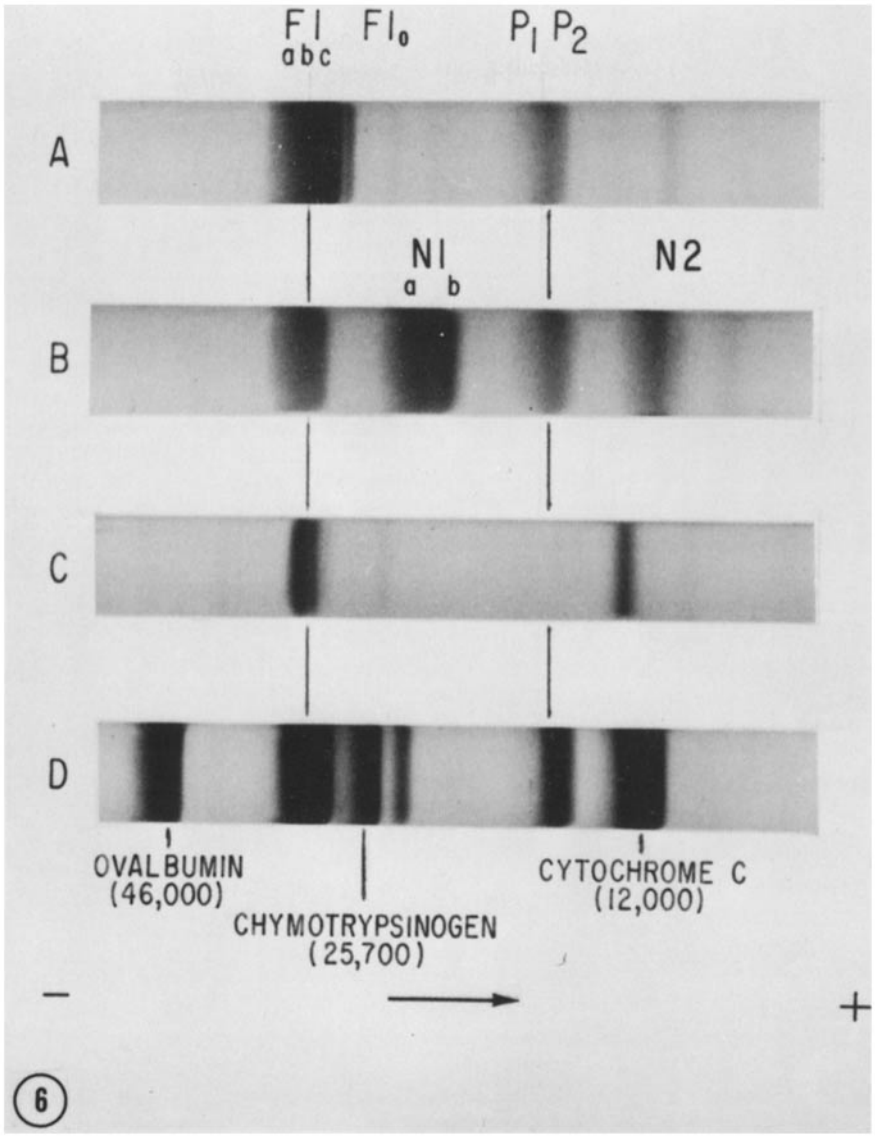

FIGURE 6 13% SDS-polyacrylamide gel electrophoretic profiles of : (*A*), 25 μg of 6% TCA-soluble protein from whole Chinese hamster cells; (*B*), 25 μg of 6% TCA-soluble proteins after NBS cleavage; (*C*), 6% TCA-soluble proteins (20 μg) from a 100,000 *g* pellet (microsomal) fraction of Chinese hamster cells. Note that F1 has its counterpart in the microsomal fraction (26); (*D*), mixture of 6% TCA-soluble proteins and three molecular weight marker proteins.

all cases the amount of ^{32}P in N2 fragments is between 26 and 30% for both in vivo and in vitro phosphorylated F1. This value is very nearly the same as that found in N2 after long-term labeling (Fig. 7). Hence, there appears to be no asymmetric or selective phosphorylation of either the N1 or N2 fragment of M-cell F1.

Tryptic Phosphopeptides of 6% TCA-Extracted Phosphoproteins of Chinese Hamster Cells Phosphorylated In Vivo and In Vitro

Although it was amply demonstrated in the previous section that only F1 and P1P2 contain pro-

tein-bound phosphate, it is apparent that simple bisection of F1 with NBS does not afford sufficient resolution of individual phosphorylation sites to detect specific differences between M- and I-cell F1. Alternatively, examination of phosphopeptides of whole unfractionated F1 in a fashion exemplified by Langan (13–16) was necessary to establish such differences. The results of these experiments are summarized in Figs. 9 and 10.

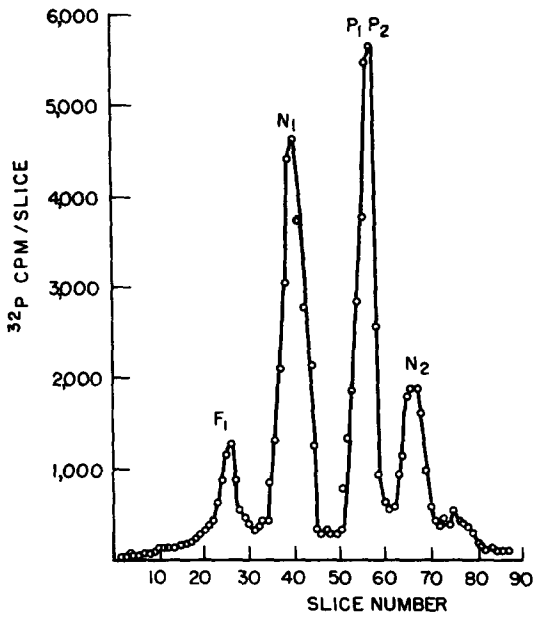

FIGURE 7 Distribution of ^{32}P in phosphoproteins of a 6% TCA extract of Chinese hamster cells prelabeled with [^{32}P]phosphoric acid for 24 h. The sample has been cleaved with NBS as in Fig. 6 *B*.

Because 6% TCA extracts of Chinese hamster cells contain both F1 and P1P2, it is first necessary to determine which tryptic phosphopeptides (p-peptides) are contributed by P1P2 and which from the total subfractions of F1. Fortunately, P1P2 is found to contribute only two major p-peptides which have an electrophoretic mobility much slower than the complex of F1-specific p-peptides (Fig. 9 [*A*]) and some minor p-peptides in the region of F1 p-peptides which would not be perceptible in total TCA-soluble proteins. Also simplifying the distinction between P1P2 and F1 p-peptides is the earlier observation (Fig. 8) that P1P2 is not phosphorylated in vitro by the KII enzyme. The P1P2-specific p-peptides shown in Fig. 9 *A* were derived by taking a 6% TCA extract of whole cells in vivo labeled for 24 h and subjecting it to electrophoresis on 13% SDS gels. F1 and P1P2 bands were manually cut and eluted from the gels in 1% SDS for 48 h at 37°C. After addition of 50 μg of cold carrier F1, the eluted protein was precipitated with TCA, washed with acetone to move bound SDS, and trypsin digested as described in Methods.

Direct examination of 6% TCA-extracted pro-

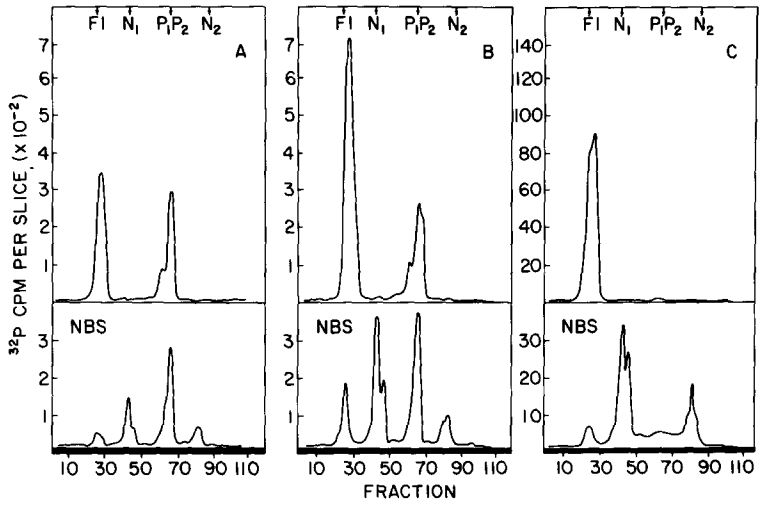

FIGURE 8 13% SDS-polyacrylamide gel electrophoretic profile of 6% TCA extracts of whole Chinese hamster cells pulse labeled with ^{32}P for 3 h or phosphorylated in vitro. The upper part of each panel is uncleaved and the lower panel is after NSB cleavage of one-half of the sample. (A) 6% TCA extract of I cells, 50 μg; (B) 6% TCA extract of M cells from the same population after 4 h of vinblastine arrest, 50 μg; (C) 25 μg of 6% TCA extract phosphorylated in the standard in vitro reaction mixture with KII.

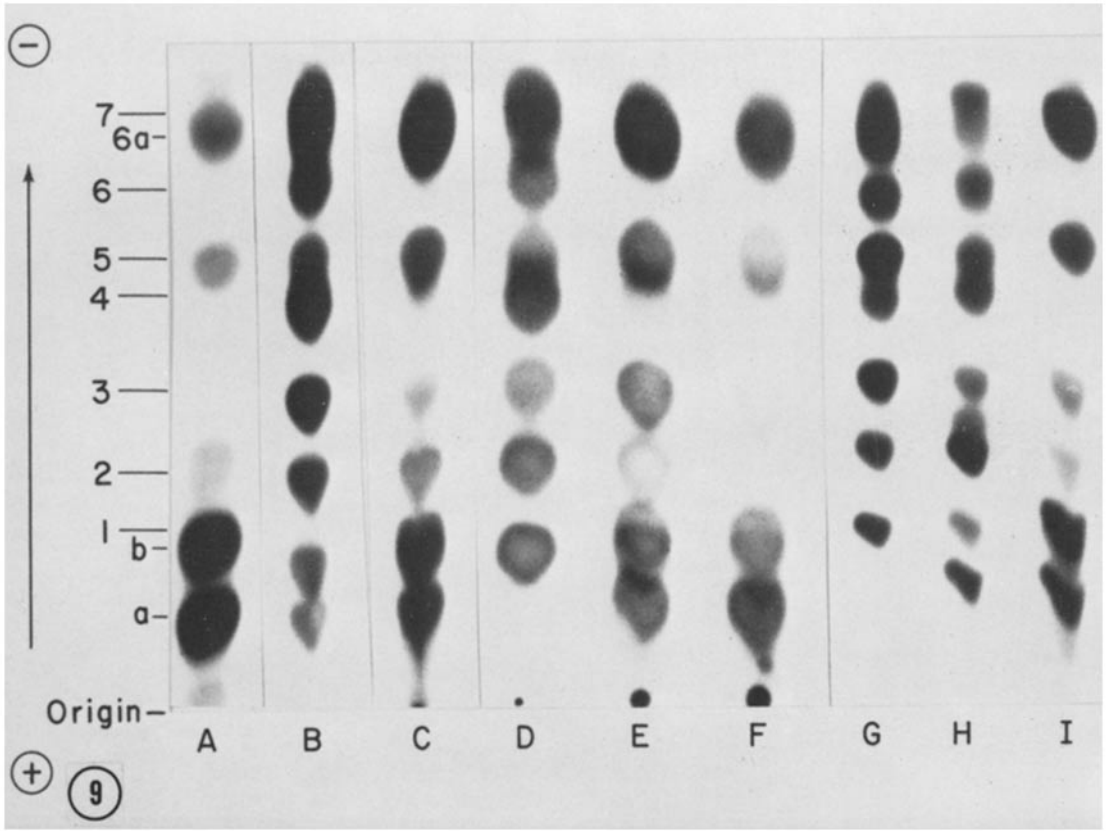

FIGURE 9 One-dimensional tryptic-phosphopeptide maps of 6% TCA-extracted proteins derived by in vivo phosphorylation of Chinese hamster and HeLa-S3 cells. (*A*), phosphorylated P1P2 obtained by elution from SDS gels; (*B*), 3 h pulse-labeled Chinese hamster M cells; (*C*), 3 h pulse-labeled I cells from the same population as *B*; (*D*), 2 h pulse-labeled Chinese hamster M cells; (*E*), I cells; (*F*), R cells not treated with vinblastine; (*G*), F1 phosphorylated in vitro with the Chinese hamster cell phosphokinase II; (*H*) and (*I*), HeLa-S3 M and I cells, respectively, from a 3 h pulse-labeled population.

tein from whole in vivo-labeled M and I cells was then performed. Chinese hamster cells were labeled with ^{32}P for either 2 or 3 h during collection of M cells in vinblastine. A control culture of R cells not treated with vinblastine was included for comparison with what is being called I cells. I cells are in reality that portion of the R cells not shaken from the vinblastine-treated monolayer during collection of M cells. Most significantly and of primary concern is a radical difference in the p-peptide pattern observed in M vs. I cells (Fig. 9 *B–C* and *D–E*).

For convenience, these major p-peptides have been designated in order of increasing mobility 1-7 and the P1P2 p-peptides as a, b. P-peptide b partially overlaps p-peptide 1 in these one dimensional maps. All major p-peptides found in I cells correspond to ones found in M cells except for peptide designated 6a, which is intermediate in mobility to p-peptides 6 and 7.

Because each sample being compared in a given map has been adjusted to equal total ^{32}P activity, the difference in the M and I cell pattern is attributable to an M cell-specific pattern of higher ^{32}P specific activity being superimposed on and obscuring the I pattern. This obscuring effect is best recognized by noting that the p-peptides a, b, and 6a which are prominent in the I sample are almost completely obscured in the M sample. This is particularly evident in the 2 h pulse-labeled maps compared in Fig. 9 *D–F*. This same type of change in the p-peptide pattern, although slightly different, is also seen with HeLa S3 cells pulse labeled for 3 h during collection in vinblastine (Fig. 9 *H*, *I*).

Knowing from the previous gel filtration char-

acterization of Chinese hamster cell phosphokinases (Fig. 1) that KII is the activity contributing to the high overall phosphokinase activity in M cells, it was obvious to examine its in vitro p-peptide pattern in comparison with KI and other in vivo derived patterns. Fig. 9 *G* shows that KII phosphorylates p-peptides *1–7*, exclusively. That these seven phosphorylation sites correspond to those predominating in the in vivo-labeled M cells is evident. A pattern identical to that seen in Fig. 9 *B* or *D* can be reconstructed by mixing in vivo-phosphorylated I-F1 with three times the ^{32}P activity from F1 phosphorylated in vitro with KII. This argues for the conclusion that KII is responsible for the in vivo pattern in pulse-labeled M cells and that F1 is its natural substrate in vivo.

Fig. 10 *A–C* demonstrate that KI, as distinct from KII, phosphorylates p-peptide 3 predominantly. Furthermore, KII of either M or I cells gives the same p-peptide pattern which is clearly distinct from patterns derived from KI. Strict identity between these in vitro-derived p-peptides is consistent with the previous observation that KII from M and counterpart I cells has a similar substrate profile and K_m for ATP. Such a marked similarity between KII from both cell types indicates that the same enzyme is present in a random population and not restricted to M cells.

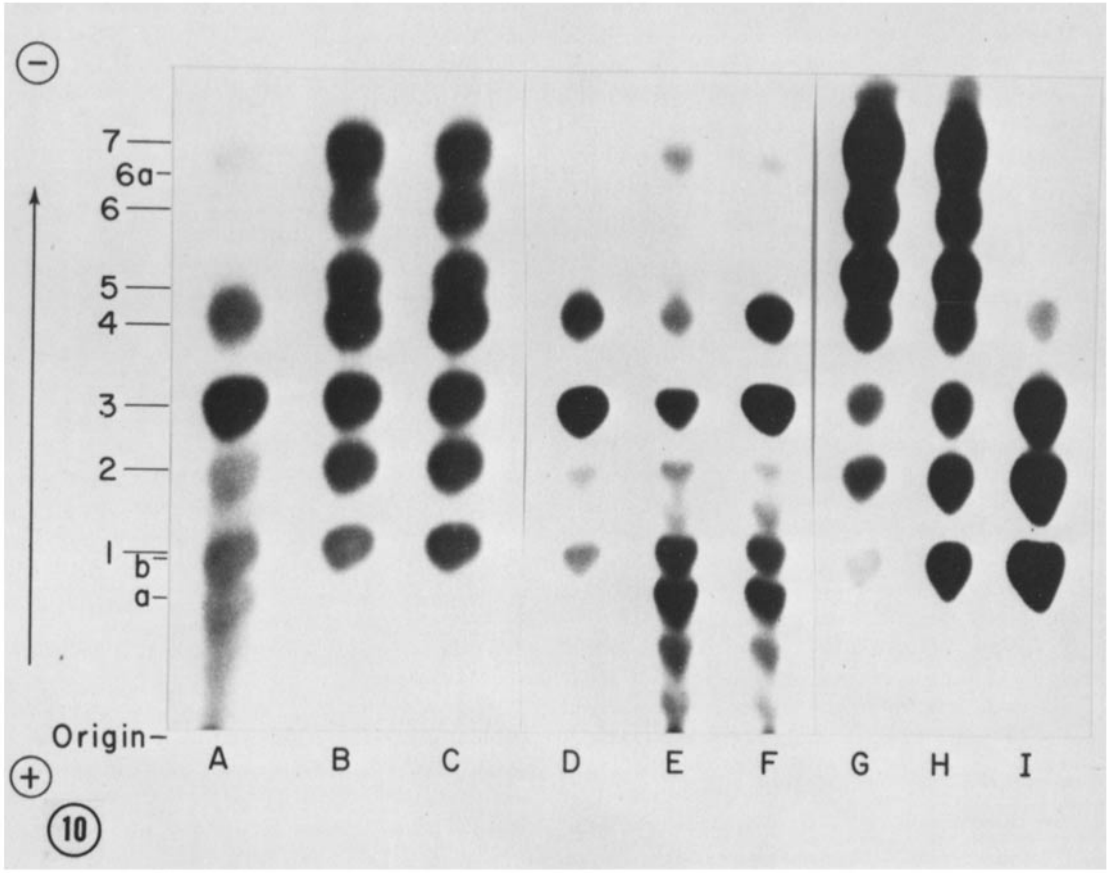

FIGURE 10 One-dimensional tryptic-phosphopeptide maps of F1 phosphorylated in vitro with either phosphokinase I (KI) or phosphokinase II (KII) under various conditions. (*A*), KI under standard conditions at pH 9.0; (*B*), KII from M cells; (*C*), KII from R Chinese hamster cells; (*D*), KI at pH 9.0; (*E*), KI at pH 7.0; (*F*), KI at pH 7.0 + cAMP at 2×10^{-6} M. The last three maps are of Chinese hamster cell F1 phosphorylated with KII at pH 9.0. A portion of the F1 was cleaved with NBS and the fragments separated on a Sephadex G-100 column (17). An uncleaved control aliquot was subsequently run on the same column. (*G*), N1 fragment + uncleaved F1 contamination; (*H*), uncleaved F1 control; (*I*), N2 fragment.

Additional factors influencing the specificity of KI and KII for F1 phosphorylation sites have also been examined. KII, whose activity for phosphorylation of F1 diminishes at low pH and high NaCl concentration does, however, yield the same qualitative p-peptide pattern under these conditions. The KII pattern is also unaffected by cAMP (2×10^{-6} M), treatment of the product with hydroxylamine, presence or absence of Triton X-100 in the reaction mixture, and the previous dephosphorylation of the native F1 substrate with alkaline phosphatase. KI, on the other hand, as shown in Fig. 10 *D–F* exhibits a preference for P1P2 sites, particularly at low pH. When stimulated with cAMP, KI does so at sites 3 and 4 of F1 but seemly not at P1P2 sites. At pH 9.0 KI has less preference for P1P2 sites and is not stimulated by cAMP. Although these observations are peripheral to the identification of the enzyme responsible for F1 phosphorylation in M cells, they serve to illustrate that exponentially growing animal cells possess at least two major phosphokinases with widely divergent properties, but potentially a common substrate in vivo.

Lastly, as shown in Fig. 10 *G–I*, is the observation that p-peptides 1, 2, and 3 reside in the N-terminal N2 fragment of Chinese hamster F1. A minor p-peptide migrating in the region of peptide 4 is also recovered with the N2 fragment after in vitro phosphorylation of F1 with the KII enzyme. It may arise from NBS cleavage of the F1 molecule at a tryptic peptide which includes the internal tyrosine residue or is a minor N2 peptide not seen in whole F1 patterns. Despite the fact that the N1 (C-terminal) fragment is incompletely separated from uncleaved F1 on G-100 gel filtration (6), it is surmised by subtraction of the N2 peptides from the whole F1 pattern, that p-peptides 4–7 reside in the N1 fragment. Slow cathodal migration of p-peptides 1, 2, and 3 is consistent with the fact that the N2 fragment is rich in acidic and hydrophobic residues compared with the highly basic N1 fragment (17). Taken together, these considerations indicate that F1 phosphorylation with the KII enzyme, and thus in M cells, occurs in both the N1 and N2 fragments of F1.

DISCUSSION

The present experiments serve to identify the F1-histone phosphokinase of M-arrested Chinese hamster cells. It is shown that total phosphokinase activity can be separated into two major activities by gel filtration. One activity (KI) is >250,000 da, cAMP dependent, slowly turned over, and specific for a major phosphorylation site in the N-terminal fragment of F1 histone. The other activity (KII) is ~90,000 da, cAMP independent, rapidly catabolized, specific for seven major sites in the F1 molecule, and the predominant activity in M-arrested cells. KII, although of very high specific activity in M cells, is similar in all respects examined to its counterpart enzyme found in interphase and randomly growing cells.

These findings compliment and extend two previous studies on the phenomenon of F1-histone phosphorylation in M-arrested animal cells (4, 5). Based on the degree of F1 phosphorylation and the ease of dissociation of M-cell F1 from nuclear DNA it was previously suspected that M-cell F1 phosphorylation must be multiple and unique (4). Tryptic-phosphopeptide maps of Fig. 9 confirm that in vivo M-cell F1 phosphorylation is unique compared to I-cell phosphorylation. That the M-cell F1 molecule is multiply phosphorylated also seems likely. This is surmised from the extent of the electrophoretic mobility shift in urea-acetic acid polyacrylamide gels (4) and the finding here of seven major p-peptides derived from in vivo labeling experiments.

Present detailed knowledge of individual phosphorylation sites in F1 of other mammalian systems comes largely from the work of Langan (14, 15) with rat liver and calf thymus. In vitro, calf thymus F1 can be phosphorylated at a major site A in the N2 fragment and a minor site B in the N1 fragment. Each site is acted upon by a separate enzyme, one being cAMP stimulated and the other not. In vivo, the A site is phosphorylated in hormone-treated rats, presumably by the cAMP-stimulated phosphokinase having specificity for site A. Recognizing that any given cell type or tissue has multiple phosphokinases (15, 18, 19) there is reason to expect that in vivo p-peptide patterns are a collection of the action of several phosphokinases. In the case of M cells, it appears that KII is a predominating activity. Because KII cannot be subfractionated, as indicated in Fig. 2, there is reason to assume that KII is a single entity able to phosphorylate seven sites. Equation of in vivo and in vitro p-peptide patterns in the same cell type is potentially complicated by nonrandom phosphate removal by protein phosphatases and possible unique conformation (availability of sites) of F1 in the native complex vs. in solution. Superimpose on these variables the multiplicity of en-

zymes able to act on F1 and the inherent heterogeniety of F1 with respect to subfractions and one is struck by the fact that in vivo-phosphorylated M-cell F1 is so closely mimicked at all seven sites by the KII enzyme in vitro. This evidence is convincing that F1 is one of the natural substrates for Chinese hamster cell KII in vivo. Other phosphorylated sites seen after long term ^{32}P labeling and during short pulses of I cells probably arise from the action of other phosphokinases as yet undetected in vitro because of inappropriate reaction conditions or lack of essential cofactors. During long labeling periods, the net p-peptide patterns are likely to be modulated or adjusted by the action of a phosphatase selective for particular sites according to their availability during the dynamic function of F1 in the native complex. Evidence for dynami c function of posttranslational F1 phosphorylation comes from the observations of Balhorn et al. (20) that F1 histone itself turns over very little but that F1 phosphate turns over with an apparent half-life of 5 h in rat hepatic tissue culture cells.

The finding illustrated in Fig. 10 *G–I*, that M-cell F1 phosphorylation occurs at sites in both the N1 and N2 fragments, argues against a possible asymmetric (polar) distribution of phosphate. Asymmetric distribution in the M-cell N1 fragment was expected to hint to a specific modification of the interaction of M-cell F1 with DNA. Originally, the C-terminal N1 fragment was suggested to be that highly basic two-thirds of the F1 molecule which interacts with DNA (17). Subsequent work on the primary structure of F1 has indicated that either or both basic ends (21) of F1 could be functional in this regard. Clearly, knowledge of the position of M–F1 phosphorylation sites in relation to those regions purported to interact with DNA is needed before a function in mitotic cells can be ascribed. Fasman et al. (22) have, from considerations of the ability of F1 fragments alone and in combination to distort the DNA circular dichroic spectrum, proposed that the N2 fragment, influenced by its phosphorylation, might fold back on the N1 fragment so as to modify its binding to DNA. This is not likely to be the case in M cells, for both fragments are phosphorylated to a similar extent and no distortion of the M-cell chromatin circular dichroic spectrum is found (23). Because F1 phosphorylation in the N2 fragment causes it to be less effective in causing circular dichroism changes in the DNA of artificial complexes (24) and in blocking the accessibility of deoxyribonuclease to DNA (25), it is thought that F1-histone phosphorylation could be an essential adjunct to regulation of transcription (14). Without departure from these current notions about the function of this type of F1 phosphorylation the alternative is to propose that F1 phosphorylation at the G_2-M transition is not a reflection of gene activation and increased transcription but is a unique form of F1 phosphorylation involved in some way in the characteristic structure and repressed state of metaphase chromatin.

Whatever the biological function(s) of histone phosphorylations are in general, it is clear from the present results that F1 phosphorylation in M-arrested cells is both qualitatively and quantitatively distinct from that which occurs throughout interphase of the cell cycle. Recognition of the existence of this specific phosphorylation-dephosphorylation reaction in cells traversing into and out of mitosis should be of utility in future study of the functional associations between the elements of chromatin during mitosis.

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