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Occurrence and Properties of a Chromatin-Associated F1-Histone Phosphokinase in Mitotic Chinese Hamster Cells†

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ABSTRACT: A chromatin-associated phosphokinase (ATP: histone phosphotransferase) activity having high specificity for F1 (lysine-rich) histone has been detected in mitotic (M) Chinese hamster cells. This activity, which is cyclic 3′,5′-adenosine monophosphate independent and elevated 6- to 10-fold in specific activity over that found in interphase (I) cell chromatin, is shown to account for an electrophoretic heterogeniety observed in F1 histone of mitotic cells. Control experiments have excluded the possibility that the differences in phosphokinase activity assayed in M and I chromatin are due to differential phosphatase, adenosine triphosphatase, or protease activities. F1-phosphokinase specific activity is highest in chromatin, but is also elevated in other mitotic cell fractions. This bound activity can be dissociated along with the non-histone proteins by treatment of chromatin with 0.35 M NaCl without loss of activity. Since phosphokinase activity decays with long metaphase-arrest times and in the presence of cycloheximide, it is suggested that F1 phosphokinase exists as an unstable enzyme especially active during a period of the cell cycle proximal to or during mitosis. The biological significance of this augmented phosphorylation and its relationship to cell cycle and mitotic events remain undefined.

Specific phosphorylation and dephosphorylation of histone F1, F2a2, and F3 fractions has been studied in a variety of mammalian tissues (Delange and Smith, 1971; Langan, 1971) to determine if protein modification reactions modulate gene activity by derepression. Recent comparisons have been made between phosphorylation in normal and regenerating liver (Gutierrez-Cernosek and Hnilica, 1971; Balhorn *et al.*, 1971) and between stationary and synchronously growing cultured cells (Balhorn *et al.*, 1972a,b). Their findings indicate that histone phosphorylation occurs coincident with cell growth but that it is not necessarily coupled with histone biosynthesis.

F1 (lysine-rich) and F2a2 (moderately lysine-rich) histone fractions are significantly phosphorylated in cultured animal cells. This has been shown for Ehrlich ascites (Sherod *et al.*, 1970), Chinese hamster ovary (Gurley and Walters, 1971), and HTC hepatoma cells (Balhorn *et al.*, 1972a). Sherod *et al.* (1970) and Balhorn *et al.* (1971, 1972a,b) have determined that an electrophoretic heterogeneity of F1 bands and phosphorylation of F1 molecules are coincident with the DNA synthetic (S) phase of the cell cycle.

Gurley and Walters (1971), however, have noted that Chinese hamster cell F1 phosphorylation is sensitive to X

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irradiation and correlates with F1 turnover rather than DNA synthesis *per se*. Likewise, Shepherd *et al.* (1971a) could find no temporal correlation between DNA synthesis and metabolic incorporation of phosphorus into histone fractions of synchronized Chinese hamster cells. This finding, although open to question (Balhorn *et al.*, 1972b), coupled with an apparent constant specific activity of F1 phosphokinase(s) throughout interphase of the cell cycle (Shepherd *et al.*,1971b) has encouraged the notion that F1 phosphorylation is related to some cellular event coincident with, but not directly related to, DNA synthesis.

Recently we reported a greatly augmented F1-histone phosphorylation occurring in mitotic Chinese hamster cells (Lake *et al.*, 1972). In the present report, we further characterize the occurrence of this event and describe properties of an augmented chromatin-associated F1-phosphokinase activity which is apparently responsible for this phosphorylation.

Materials and Methods

Preparation of Metaphase and Interphase Cells. A clonal line of Chinese hamster cells (V79-589FR) with an aneuploid chromosome number of 23 and a generation time of ~13 hr (G1, 4 hr; S, 6 hr; G2, 3 hr; M, 0.5 hr) was grown in suspension in Eagle 2 medium with spinner salts plus 10% fetal calf serum (Industrial Biological Laboratories, Rockville, Md). Cells were seeded in large roller bottles, 650-cm^2 surface area, in Tricine-buffered Eagle's 2 (Tricine-EM) containing 10% fetal calf serum at 10^8 cells/bottle. After 18-hr exponential growth, 0.1 μg/ml of vinblastine (Velban, Eli-Lilly Co.) was added. After 4-hr treatment, approximately 30% of the cells is accumulated at metaphase. Metaphase (M) cells were shaken off in 50 ml of warm Tricine-EM. Remaining interphase (I) cells were rinsed vigorously to remove any residual M cells and gently removed by rolling 2-mm glass beads in the bottle. The per cent M cells was typically 95% as checked by direct Acetoorcein staining. As is apparent from the generation cycle times, a 4-hr arrest depletes the population of G1 stage cells leaving predominantly S and G2 cells in the interphase population.

Deoxynucleoprotein Preparation and Cell Fractionation. Cells were washed by centrifugation at 200*g* for 10 min in Tris-buffered isotonic saline and adjusted to equal packed cell volumes. The M and I cells were then swollen for 5 min at 0° in hypotonic reticulocyte standard buffer (RSB, 10 mM Tris-HCl–10 mM NaCl–15 mM $MgCl_2$, pH 7.6), then diluted 1:1 with 0.5 M sucrose in water. After low-shear homogenization, the nuclear and cytoplasmic fractions were separated by centrifugation at 2500*g* for 10 min. The nuclear fraction was re-homogenized once in RSB (in some experiments containing 0.1% Triton X-100) and resuspended in 10 mM Tris–1 mM Na_2EDTA (pH 7.6). After homogenization in a Teflon-pestle Potter-Elvehjem-type homogenizer at 12,000 rpm, the suspension was clarified at 1000*g* for 10 min. The supernatant chromatin was pelleted at 27,000*g* for 15 min in a Sorvall SS-34 head. This unsheared chromatin had protein:RNA:DNA mass ratios of 2.1–2.3:0.01–0.04:1 by direct analysis and did not differ whether from M or I cells. When used as enzyme source the pellet was resuspended in 1 mM Tris–HCl–6 mM $MgCl_2$–14 mM β-mercaptoethanol–5% glycerol (pH 7.6). In order to solubilize the material, this mixture was treated at room temperature for 10 min with 1 μg/ml of DNase I (Worthington Biochemicals, RNase free).

Postnuclear fractions were separated by differential centrifugation. A mitochondrial fraction was obtained by centrifugation at 27,000*g* for 20 min; a microsomal fraction by centrifugation of this supernatant at 100,000*g* for 2 hr. Supernatant proteins were precipitated at 60% ammonium sulfate saturation and pelleted at 10,000*g* for 10 min. Cell fractions were resuspended in RSB containing 14 mM β-mercaptoethanol prior to storage at −20° and protein determination by the assay of Lowry *et al.* (1951).

Chinese Hamster Cell F1 Histone. The term F1 is used throughout to mean lysine-rich histone I that is soluble in 6% trichloroacetic acid. The preparations of Chinese hamster F1 that are obtained using 6% trichloroacetic acid consist of two major and two minor lysine-rich components and a non-lysine-rich subcomponent (manuscript in preparation). Fresh whole cells, isolated nuclear fraction, or purified chromatin were extracted at 0° by adding 12% trichloroacetic acid to an equal volume of the nucleoprotein in distilled water. After 30-min intermittent homogenization in a Dounce-type homogenizer the extract was centrifuged at 2000*g* for 15 min. The proteins contained in the supernatant fluid were precipitated by adding 100% trichloroacetic acid to a final concentration of 25%. The trichloroacetic acid precipitate was dissolved in a small volume of water and precipitated with ten volumes of acid–acetone (0.5% v/v concentrated HCl in acetone) and washed twice with acetone. F1 preparations labeled with radiophosphorus were routinely redissolved in 1 N NaOH containing 5 mM sodium pyrophosphate ($NaPP_i$) and 1 mM dibasic sodium phosphate (NaP_i) at room temperature to remove labeled phosphate as described for the kinase assay (see below).

[^{14}C]Lysine- and [^{3}H]lysine-labeled F1 were obtained by growing Chinese hamster cells in suspension culture in Eagle's 2 with one-tenth the normal lysine level and labeling for 40 hr with L-[^{14}C]lysine or L-[^{3}H]lysine. Specific activity of the F1 extract was 13,900 cpm/μg of [^{3}H]F1 and 6610 cpm/μg of [^{14}C]F1.

F1-Histone Phosphokinase Assay. To define the optimum conditions for detection of chromatin-associated F1 phosphokinase, the effect of the preincubation temperature (for 30 min), pH, and the concentrations of Mg^{2+}, F1, and ATP were determined (Figures 1 and 2). The final standard incubation mixture contained, in a final volume of 0.25 ml, 40 mM Tris-HCl (pH 8.6), 10 mM $MgCl_2$, 0.1% Triton X-100, 50 μg of F1 histone, 20 nmol of ATP containing a known activity of [γ-^{32}P]ATP (New England Nuclear Corp.), and 50 μg of chromatin enzyme protein. When indicated, cyclic 3′,5′-adenosine monophosphate (cAMP, Calbiochem) at $2–5 \times 10^{-6}$ M and theophylline at 2 mM were included. The reaction was stopped after 10 min at 36°, the time after which γ-phosphate (γ-P_i) incorporation reaches a plateau (Figure 3), by chilling and adding trichloroacetic acid to 20% final concentration. All trichloroacetic acid solutions contained 5 mM $NaPP_i$ and 1 mM NaP_i. After centrifugation at 2000*g* for 15 min, the pellet was resuspended for 2 min at room temperature in 0.2 ml of 1 N NaOH containing 5 mM $NaPP_i$ and 1 mM NaP_i. After reprecipitation at 20% trichloroacetic acid, the volume was brought to 2 ml with 20% trichloroacetic acid and the precipitate was collected and washed at room temperature on 0.45-μ filters (HAWP, Millipore Filter Corp.). ^{32}P radioactivity was counted in a Triton X-100 toluene-based scintillation fluid [toluene–Triton X-100–Liquifluor (New England Nuclear)–water, 660:300:40:100, v/v] in a Beckman LS-250 spectrometer. Incorporation of phosphorus is calculated from the specific activity of added [γ-^{32}P]ATP and expressed as pmoles of γ-P_i transferred to 50 μg of F1 acceptor protein. Under these conditions

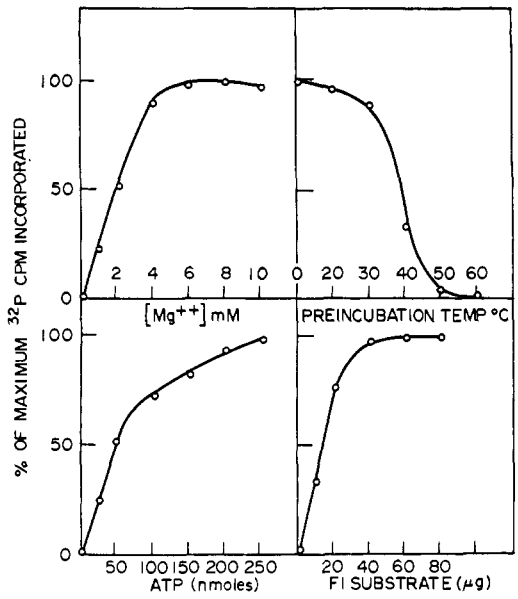

FIGURE 1: Effect of varying Mg^{2+}, ATP, F1-acceptor protein, and preincubation temperature (30 min) on Chinese hamster cell chromatin-associated F1-phosphokinase activity.

a direct correspondence between micrograms of chromatin protein added and incorporation of γ-P_i into F1 is observed as depicted in Figure 4.

Special problems were encountered when using F1 as acceptor protein. As was previously noted (Balhorn *et al.*, 1971), F1 avidly binds P_i, ADP, ATP, and PP_i such that they are not easily washed from the protein with repeated trichloroacetic acid or acetone precipitations. For this reason, F1 must be washed with 1 N NaOH at least once to effectively exchange out these nonspecifically bound anions and insoluble ATP-Mg^{2+} complexes (Greenway, 1972). Control experiments with ^{32}P-labeled F1 indicated that true covalently linked phosphate monoesters with serine and threonine are not hydrolyzed by such treatment for up to 1 hr even at 37°. NaOH (1 N) is also necessary to effectively dissolve Triton X-100 which is coprecipitated with F1 from the reaction mixtures.

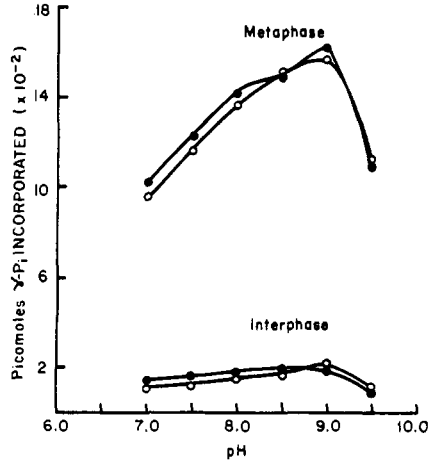

FIGURE 2: pH optimum of metaphase and interphase cell chromatin-associated F1 phosphokinase. (○) −cAMP; (●) +cAMP, 2×10^{-6} M.

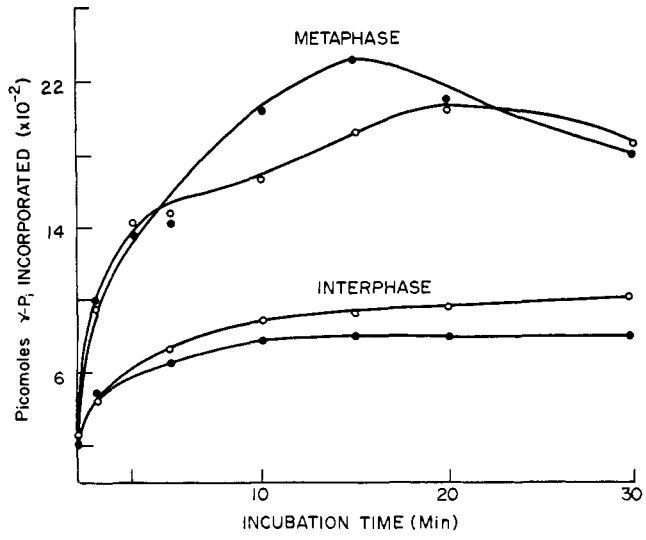

FIGURE 3: Time course of γ-P_i incorporation into F1 under standard assay conditions. (○) −cAMP; (●) +cAMP, 2×10^{-6} M.

F1 was reisolated from reaction mixtures by initial trichloroacetic acid precipitation at 6% trichloroacetic acid to remove non-F1 proteins, followed by the steps as indicated above to isolate F1.

Polyacrylamide Gel Electrophoresis. Short (8 cm) and long (25 cm) 15% acrylamide gels with 6.25 M urea and 0.9 N acetic acid (pH 2.7) were run according to Panyim and Chalkley (1969). Histone samples were reduced with 0.5 M β-mercaptoethanol before electrophoresis. Gels were stained in 0.1% Amido Black in 7.5% acetic acid–10% methanol and destained electrophoretically. Radioactivity was determined by slicing Dry-Ice frozen gels in a Mickle-type slicer. Slices (1 mm) were solubilized at 60° for 5 hr in 30% H_2O_2 and counted in Triton X-100 toluene-based scintillation fluid. Radioactivity in triple-labeled samples was computed and

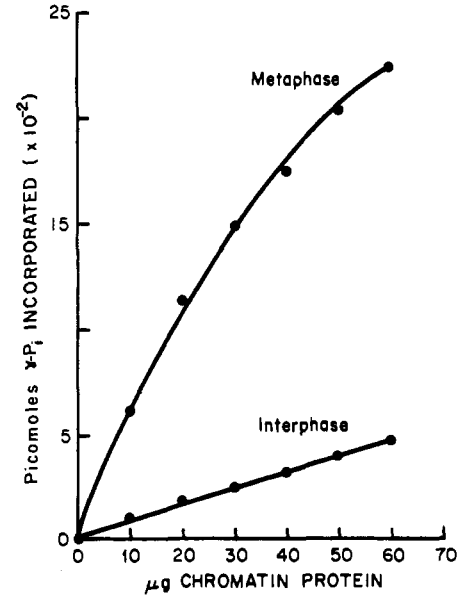

FIGURE 4: Correspondence between amount of chromatin-associated enzyme added and incorporation of γ-P_i from ATP under standard assay conditions.

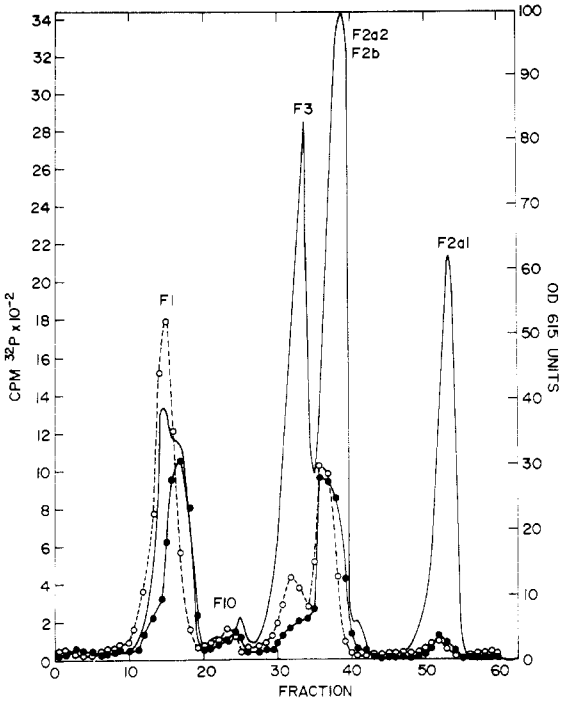

FIGURE 5: Distribution of ^{32}P in total histones of metaphase (○) and interphase (●) cells after a 3-hr pulse with [^{32}P]phosphoric acid. The continuous line is a densitometer tracing of the metaphase histone sample. ^{32}P profile is derived from parallel urea–acetic acid–polyacrylamide gels.

plotted using a program developed by Dr. Edwin Sebring of this laboratory.

Thin-Layer Chromatography of Adenine Nucleotides. ATP, ADP, and AMP were separated by thin-layer chromatography on polyethyleneimine (PEI)-cellulose (J. T. Baker, Co.) in 0.2 M LiCl–0.75 M acetic acid as described by Randerath (1968). cAMP and 5′-AMP were separated on PEI-cellulose thin layers developed in 0.2 M LiCl at pH 5.0. [8-^{3}H]-cAMP (Schwarz-Mann) was used as a marker to estimate recovery of cAMP and conversion to 5′-AMP.

Column Chromatography. F1 subcomponents were chromatographed on a 0.9 × 15 cm Amberlite IRC-50 (Bio-Rex 70, Bio-Rad Laboratories) using a 140-ml linear gradient of 8.5–14% guanidinium chloride in 0.1 M sodium phosphate buffer (pH 6.8) as described by Hohmann and Cole (1971).

Results and Discussion

Previous indirect evidence for augmented phosphorylation of F1 histone in mitotic cells was based on the existence of an alkaline phosphatase sensitive shift to lower mobility on urea–acetic acid–polyacrylamide gels (Lake *et al.*, 1972). Direct evidence for this conclusion has now been obtained by *in vivo* labeling of Chinese hamster cells with ^{32}P and by duplicating the observed mobility shift with *in vitro* enzymatic phosphorylation.

Randomly growing Chinese hamster cell monolayers were rinsed once with phosphate-free Tricine-EM and incubated for 3 hr in phosphate-free Tricine-EM supplemented with 10% dialyzed fetal calf serum, 0.05 mCi/ml of carrier-free $H_3{}^{32}PO_4$, and 0.1 μg/ml of vinblastine. Under this labeling and metaphase cell collection regimen (with a G2 phase of ~3 hr), we assume that no subsequently harvested M cells will have undergone late S-phase labeling. The small number of late S-phase cells able to traverse G2 in less than 3 hr would be labeled from a nascent [^{32}P]ATP pool. Shepherd *et al.* (1971a) have estimated the time for the phosphate pool to reach constant specific activity to be 1 hr after addition of ^{32}P. Interphase and metaphase cells were separated as described in the Methods section. Equal quantities (60 μg) of each histone sample extracted from the respective chromatins were loaded on urea–acetic acid gels. After staining and densitometer tracing the ^{32}P was counted in gel slices. Figure 5 shows the ^{32}P distribution in total histones superimposed on the densitometer tracing of metaphase histones. In agreement with previous reports (Gurley and Walters, 1971; Balhorn *et al.*, 1972a) ^{32}P is found in F1, $F1_0$, F2a2, and F3. But, the incorporation into metaphase F1 under these labeling conditions is approximately twice that into interphase cell F1. Further, ^{32}P in the metaphase F1 is associated primarily with the back (slower mobility) region of the F1 band. This indicates that protein phosphorylation may be responsible for the mobility shift seen in mitotic cell F1 and that F1 and F3 are the only histones exhibiting the enhanced phosphorylation.

Not knowing whether the observed mobility shift was due solely to phosphorylation it was necessary to duplicate the same shift by *in vitro* phosphorylation with an F1 phosphokinase. This was done as depicted in the results shown in Figure 6 by first dephosphorylating [^{14}C]lysine F1 and [^{3}H]lysine F1 with *E. coli* alkaline phosphatase. Half of the dephosphorylated ^{14}C-labeled F1 was then used as acceptor protein in a standard *in vitro* assay mixture (described in Methods section) with F1 phosphokinase from Chinese hamster metaphase chromatin. The reisolated ^{32}P, ^{14}C-labeled F1 was then coelectrophoresed with marker ^{3}H-labeled F1 on 25-cm urea–acetic acid gels. As seen in panel A of Figure 6 the dephosphorylated F1 molecules exhibit the same mobilities, but as depicted in panel B, the ^{32}P, ^{14}C-labeled F1 migrate more slowly than the parent ^{3}H-labeled F1. The heterogeneity of the phosphorylated F1 may result from having amounts of multiply *vs.* singularly phosphorylated subcomponents of F1. Sufficient resolution of individual subcomponents of F1 is not achieved when slicing the gels. This result, however, confirms that phosphorylation from ATP can produce the mobility shift observed in F1 of mitotic cells.

Further to insure that the ^{32}P in the F1 peak of Figure 5 was seryl or threonyl phosphate, ^{32}P-labeled F1 was selectively extracted from interphase histones using the interphase cell sample obtained by *in vivo* labeling (Figure 5) and analyzed for radioactivity in *P*-serine and *P*-threonine. The sample was hydrolyzed in 2 N HCl at 100° for 8 hr and electrophoresed on cellulose thin layers in 8% formic acid. Without correction for hydrolytic loss of *P*-serine, 20% of the ^{32}P was in *P*-serine, 5% in *P*-threonine and the remainder in free P_i. The extracted sample was also treated with a variety of enzymes likely to remove ^{32}P associated with nucleic acids since they may contaminate acid-extracted histones (Shepherd *et al.*, 1970). It is apparent from Figure 7 that only alkaline phosphatase removed ^{32}P from the *in vivo* labeled F1. This observation, coupled with the detection of ^{32}P in phosphoserine, establishes that protein phosphorylation is the reaction being followed. Numerous attempts to incorporate high specific activity [^{3}H]uridine or [^{14}C]thymidine into 6% trichloroacetic acid extracted F1 have been negative.

Distribution of ^{32}P in Chromatographically Resolved F1. Two major subcomponents of Chinese hamster F1 are resolved on analytical Amberlite IRC-50 columns run according to Hohmann and Cole (1971). Each of these subfractions has a unique primary structure whose chromatographic behavior

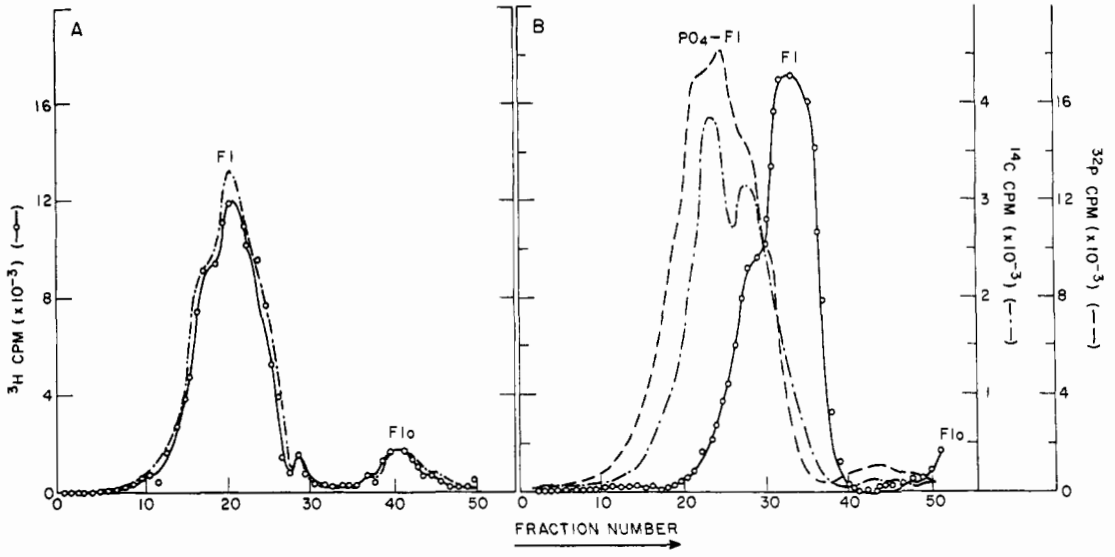

FIGURE 6: Effect of *in vitro* enzymatic phosphorylation on F1 mobility on 25-cm urea–acetic acid–polyacrylamide gels. (A) Coelectrophoresis of alkaline phosphatase dephosphorylated ^{3}H-labeled F1 and ^{14}C-labeled F1. (B) Coelectrophoresis of dephosphorylated ^{3}H-labeled F1 and dephosphorylated ^{14}C-labeled F1 after *in vitro* phosphorylation with metaphase cell chromatin-associated phosphokinase. Only that portion of the long gel containing the F1 bands has been sliced.

is not influenced by their degree of phosphorylation (Langan *et al.*, 1971) or association with RNA (Evans *et al.*, 1970). A double-label experiment with ^{32}P and ^{33}P was used to test the possibility that metaphase F1 phosphorylation occurs preferentially in one of these chromatographic subcomponents. Separate cultures of randomly growing cells were labeled with ^{32}P or ^{33}P for 3 hr during vinblastine arrest as described in the experiment of Figure 5. After harvesting metaphase and interphase cells from each population, the extracted metaphase F1 was mixed with the interphase F1 of opposite phosphorus label. The specific activity of metaphase F1 was twice that of the counterpart interphase sample. These reciprocal mixtures were added to 0.5 mg of cold carrier F1 and chromatographed. One of these chromatograms is shown in Figure 8; the ^{32}P to ^{33}P ratio in the eluted peaks was constant. If selective phosphorylation of one of the F1 subcomponents had occurred in metaphase cells, as much as a twofold change in the ratio would be expected since the phosphate specific activity in the metaphase F1 part of the reciprocal mixture was twofold

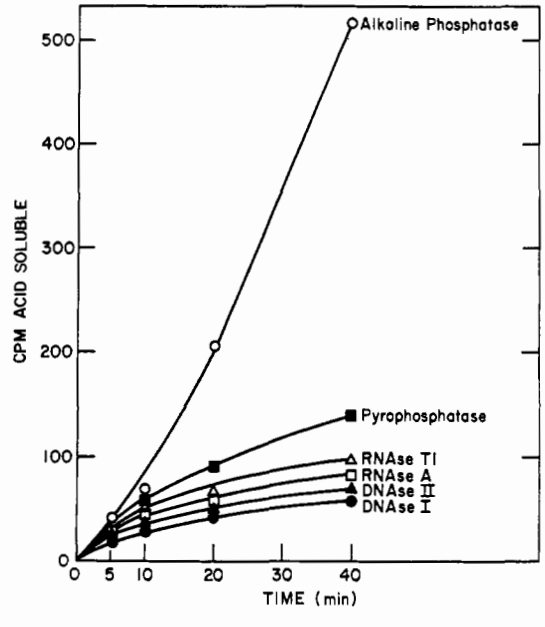

FIGURE 7: Enzymatic release of ^{32}P counts per minute from interphase cell F1 which had been labeled *in vivo* for 3 hr. ^{32}P-labeled F1 (50 μg) was added to 1 ml of each buffer containing 20 μg/ml of enzyme; after the indicated times at 36° 0.2-ml aliquots were removed and precipitated at 25% trichloroacetic acid and acid-soluble counts were determined. (○) *E. coli* alkaline phosphatase 0.1 M in Tris (pH 8.0); (■) *Crotalus adamanteus* venum, nucleotide pyrophosphatase, 0.01 M Tris–50 mM Mg^{2+} (pH 7.4); (●) DNase I, 0.1 M acetate (pH 6.5)–5 × 10^{-2} M Mg^{2+}. (▲) DNase II, 0.1 M acetate (pH 5.0)–1 × 10^{-4} M Mg^{2+}. (△, □) RNase T1, A, 0.01 M Tris (pH 7.5)–1 mM EDTA; negative control, 0.1 M Tris (pH 8.0)–0.1 M acetate (pH 5.0).

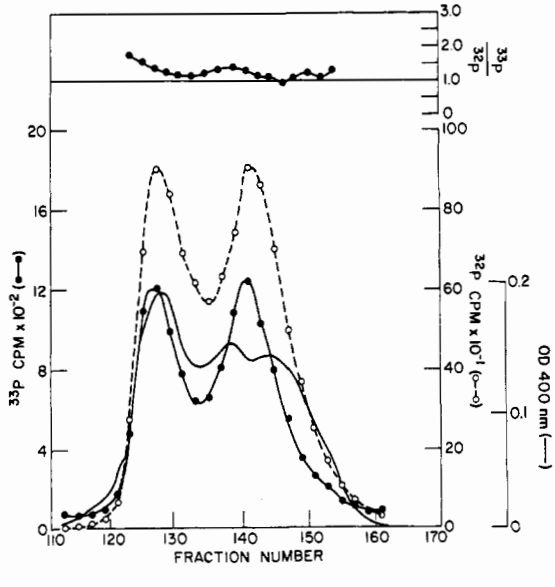

FIGURE 8: Cochromatography of ^{32}P-labeled metaphase cell F1 and ^{33}P-labeled interphase cell F1 on a Amberlite IRC-50 column. The continuous solid line is the trichloroacetic acid turbidometeric elution profile of carrier F1. Phosphorus-labeled F1 was derived as described in the text.

TABLE I: Subcellular Location of F1 Phosphokinase(s) in Metaphase (M) and Interphase (I) Chinese Hamster Cells.[a]

Cell Fraction	Total Protein (mg)		F1-Phosphokinase Sp Act. (pmoles of γ-P_i/50 μg of Enzyme Protein)			
			M		I	
	M	I	−cAMP	+cAMP[d]	−cAMP	+cAMP[d]
Total homogenate	20.0	12.4[c]	595	592	319	332
Chromatin (nuclear)	1.50 (7.5)[b]	2.00 (16.0)	1920	1755	239	229
Mitochondrial	2.50 (12.5)	1.55 (12.5)	412	404	254	240
Microsomal	3.25 (16.2)	1.00 (8.1)	573	563	322	322
Cytosol	2.00 (10.0)	0.85 (6.9)	488	489	245	265
Total (%)	46.2	42.5				

[a] Metaphase and interphase Chinese hamster cells were harvested after 4-hr vinblastine arrest, adjusted to approximately equal packed cell volumes, and fractionated as described in Methods. Kinase assay was at pH 8.5 as described in Methods but with omission of Triton X-100 and addition of 2 mM theophylline. [b] Figures in parentheses indicate the per cent recovery of protein in the original homogenate after a single wash of each fraction (except cytosol which was recovered by 60% saturated ammonium sulfate precipitation) in RSB. [c] Smaller amounts of total I protein from an equal packed cell volume of M and I cells is thought to stem from the low cell density in a pack of monolayer grown cells removed from glass without trypsinization; cells are in clumps and not spherical. [d] 2×10^{-6} M.

TABLE II: Effect of Various Conditions on Assay of Chromatin-Associated F1 Phosphokinase and Phosphatase Activity.

Chromatin Enzyme Source	Reaction Conditions	Phosphokinase (pmoles of γ-P_i Incorp)		Phosphatase[a] (% ^{32}P Remaining in F1)	
		M	I	M	I
Nonarrested[b] Chinese hamster cells	Complete	865	220	90.8	83.3
4-hr vinblastine-arrested HeLa-S3 cells	Complete	2475	487		
4-hr vinblastine-arrested Chinese hamster cells	Complete	1268	222	85.5	81.7
	+cAMP	974	200		
	−Triton X-100	1058	148		
	−Triton X-100 + cAMP	1049	147	77.0	79.5
	−F1 acceptor	92	53		
	−F1 acceptor + cAMP	111	52		
	−Chromatin	1.4	1.6	100	100
	+[γ-^{32}P]GTP	422	73		

[a] 25 μg of ^{32}P-labeled F1 (580 cpm/μg) were added to each assay mixture under conditions identical with the phosphokinase assay but with ATP omitted. [b] Mitotic cells are obtained by selective detachment at approximately 6–8 hr after release from a single excess-thymidine block.

greater. By this criterion the augmented phosphorylation is uniform throughout the chromatographic subcomponents.

Subcellular Location of F1 Phosphokinase(s). Since many protein phosphokinases will use F1 histone as acceptor of γ-phosphate from ATP, it was of interest to compare metaphase and interphase cells for their phosphokinase location. Equal packed cell volumes of M and I cells were fractionated as described in the Methods section. F1 phosphokinase activity of 50 μg of each cell protein fraction was assayed with or without the addition of 5×10^{-6} M cAMP to the reaction mixture. Table I shows the distribution of activity in various cell fractions. As is expected for a cell containing spindle protein and without an organized nuclear membrane, the relative amount of protein recovered in postnuclear fractions is greater in metaphase cells. Phosphokinase-specific activity in the total cell homogenate and in all cell fractions is greater in metaphase cells as compared to interphase cells. The most striking difference is in the phosphokinase activity of chromatin-associated proteins—ninefold greater in this experiment.

Stimulation of phosphokinase activity in these crude cell fractions by cAMP was meagre (10%) and in some cases inhibitory. This cAMP independence in *in vitro* assays was not due to phosphodiesterase activity in the crude fractions, since when [^{3}H]cAMP was added in a 10-min incubation, greater than 93% of the [^{3}H]cAMP counts was recovered on PEI-cellulose thin layers as the cyclic nucleotide and not as 5′-AMP.

TABLE III: Substrate Preferences of Metaphase Cell Chromatin-Associated F1-Phosphokinase Activity.

Substrate Substituted in Std Assay	pmoles of γ-P_i Incorporated	
	−cAMP	+cAMP[a]
No substrate (endogenous)	92	90
Calf thymus histone (whole)	467	452
Calf thymus histone I (F1)	1797	1641
Calf thymus histone IV (F2al)	263	241
Chinese hamster histone (whole)	480	509
Chinese hamster histone I (F1)	1552	1704
Phosvitin	336	299
α-Casein	320	295
Bovine albumin (fraction V)	299	295
Protamine	132	102

[a] 2×10^{-6} M.

TABLE IV: Effect of Pelleting Chromatin through 50% Sucrose and 0.35 M NaCl Extraction on F1-Histone Phosphokinase Activity.

Treatment of Chromatin	pmoles of γ-P_i Incorporated			
	Metaphase Chromatin		Interphase Chromatin	
	−cAMP	+cAMP	−cAMP	+cAMP
	Part A[a]			
None	1980		368	
50% Sucrose + 0.1% Triton X-100				
Pellet	1964		393	
Pellicle	23		24	
50% Sucrose				
Pellet	1857		336	
Pellicle	37		24	
	Part B[b]			
Ammonium sulfate precipitate	835	749	195	301
0.35 M NaCl pellet	295		97	
0.35 M NaCl supernatant precipitated with ammonium sulfate	929	469	234	255

[a] Chromatin in 1 mM Tris-HCl (pH 7.6)–1 mM EDTA was layered on 5 ml of 50% sucrose solution in 10 mM Tris-HCl (pH 7.6) in $^{9}/_{16} \times 3.5$ cellulose nitrate tubes. After centrifugation at 25,000 rpm in a SW 41 rotor for 3 hr a pellicle at the top of the cushion was harvested. The pellet was dispersed with 1 μg/ml of DNase I. [b] Chromatin in RSB was brought to 0.35 M NaCl for 1 hr at 0°. The mixture was centrifuged at 10,000g for 15 min and the supernatant precipitated at 33% saturated ammonium sulfate. The residual chromatin pellet was dispersed in 1 μg/ml of DNase I.

Properties of the Chromatin-Associated F1 Phosphokinase. As is the case with ribosome-associated protein phosphokinases (Kabat, 1971), detection of chromatin-associated protein kinase activity *in vitro* is potentially complicated by the presence in the chromatin of protein phosphatases, nucleotide phosphohydrolases (ATPase), and proteases. Any of these side reactions could contribute to the marked difference in F1 phosphokinase activity assayed in M and I cell chromatin.

Table II summarizes some of the properties found for the Chinese hamster chromatin-associated phosphokinase. Again, it is seen that there is a sixfold difference in specific activity between M and I chromatin prepared in parallel from the same cell population. This difference is also manifested in chromatin prepared from nonarrested mitotic cells and from HeLa S-3 cells. Endogenous protein kinase activity is elevated in metaphase chromatin but is not sufficient to account for the great difference in the total reaction. Triton X-100 applied either as a prewash of the chromatin (not shown) or present in the reaction mixture is stimulatory for both the M and I activities. cAMP is without effect on either the endogenous or exogenous reaction. It is also observed that γ-phosphate from GTP is used at about one-third the efficiency of ATP, but that the marked difference between M and I enzyme specific activity is maintained.

F1-phosphatase activity, measured by adding ^{32}P-labeled F1 to reaction mixtures identical to those of the phosphokinase assay but without added ATP, was similar for both chromatin enzyme sources (Table II).

Relative ATPase activity of the two types of chromatin was measured as the conversion of [8-^{14}C]ATP to ADP as separated on PEI-cellulose thin layers. ATPase activity was consistently higher (40% more conversion in a 30-min incubation) in interphase chromatin but this is not enough to account for the great difference observed in phosphokinase activity.

Evidence against differential protease activity in the assay mixtures comes from the fact that *in vitro* phosphorylated and subsequently reisolated F1 exhibits no small cleavage products on electrophoresis (Bartley and Chalkley, 1970). Moreover, addition to the reaction mixtures of 0.05 M sodium bisulfite, a potent protease inhibitor (Panyim *et al.*, 1968), does not obliterate or change the F1-phosphokinase activity in M or I chromatin.

Substrate Preferences of Chromatin Enzyme. Table III shows the strong preference of the metaphase chromatin-associated phosphokinase for lysine-rich histone, whether it is homologous Chinese hamster F1 or calf thymus F1. Again the reaction is independent of added cAMP for all substrates tested.

Removal of F1 Phosphokinase from Chromatin. Pelleting chromatin through a cushion of 1.7 M sucrose removes many non-histone proteins and membranous contaminants. When this is done, as shown in Table IV, the F1-phosphokinase activity remains in the pellet, even when 0.1% Triton X-100 is included in the sucrose. If, however, the chromatin is washed with 0.35 M NaCl, a salt concentration in which the chromatin is aggregated and insoluble, most of the original activity is solubilized. Further, the original high specific activity of metaphase phosphokinase is retained in a form not stimulated by cAMP. These facts also indicate that differential activity exhibited by M and I chromatin is not due to greater avail-

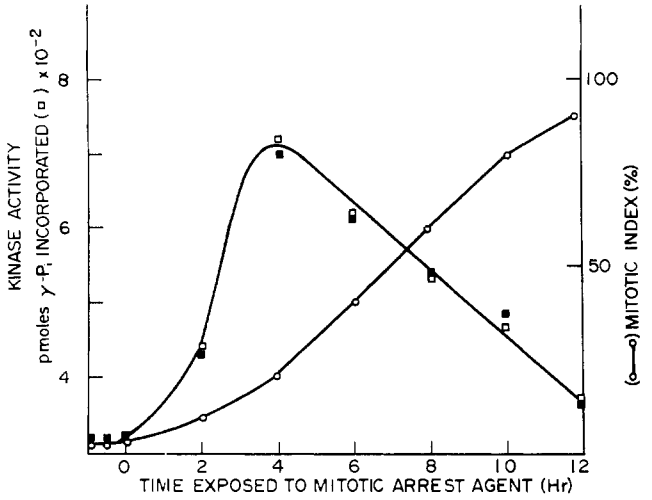

FIGURE 9: Chromatin-associated F1 phosphokinase activity in Chinese hamster suspension cultures treated at zero time with 0.1 μg/ml of vinblastine (□) or 0.06 μg/ml of Colcemid (■). Samples of 2×10^7 cells were removed at the indicated times for chromatin preparation and mitotic index determination.

ability of M kinase relative to I kinase in the chromatin-bound form.

Correlation between Mitotic Index and F1 Phosphokinase. To exclude the possibility that high phosphokinase activity was an artifact of shaking mitotic cells from monolayer cultures, the chromatin-associated activity was measured in suspension cultures treated with vinblastine or Colcemid. At various times after adding the arrest agent, aliquots were removed for determination of mitotic index and for chromatin preparation. If enhanced phosphokinase activity is due solely to the contribution of mitotic cells to the total population, there should be a direct correlation between mitotic index and kinase activity. As shown in Figure 9, chromatin F1-phosphokinase activity increases during the first 4-hr arrest and accumulation of C-metaphases, but decreases over a subsequent 8-hr period. By 12 hr, when 92% of the cells are scored as mitotic, the kinase activity has nearly returned to the level found in random exponentially growing cultures. To determine if chromatin-associated activity decays with long arrest

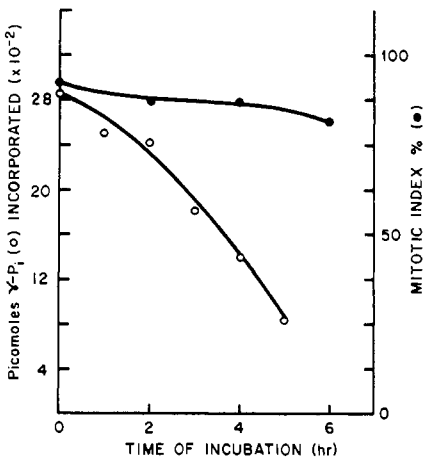

FIGURE 10: Chromatin-associated F1-phosphokinase activity in pure metaphase cells gathered for 4 hr in vinblastine and further incubated in suspension at time zero.

TABLE V: Effect of Actinomycin D and Cycloheximide on Chromatin-Associated F1-Histone Phosphokinase Activity in Suspension Cultures of Chinese Hamster Cells.

Time of Treatment (hr)	% of Initial F1-Phosphokinase Act.	
	Cycloheximide[a]	Actinomycin D[a]
0	100.0	100.0
1	78.0	91.0
2	63.1	93.1
3	50.3	96.5
4	49.8	90.1
5	50.1	88.7

[a] 2 μg/ml.

times, 4-hr-arrested metaphase cells were obtained by selective detachment from monolayer and further incubated in suspension in the presence of vinblastine to maintain arrest. As shown in Figure 10, F1 phosphokinase declines over a subsequent 5-hr incubation period, despite maintenance of metaphase chromosome condensation. Decay with long arrest times points up the unstable and dynamic nature of this high activity and eliminates a possible need for continued phosphokinase activity for maintenance of chromosome condensation. Whether dephosphorylation of F1 *in vivo* corresponds to this decay of phosphokinase activity has not been established.

As an adjunct to these experiments showing decay of chromatin phosphokinase activity with long metaphase arrest times, the effect of actinomycin D and cycloheximide was examined. The results listed in Table V indicate that maintenance of F1-phosphokinase activity in random cell chromatin (equivalent to I chromatin) is dependent on continued protein synthesis but relatively unaffected by actinomycin D. Chromatin-associated activity dropped to 50% of its initial activity within 3 hr in the presence of cycloheximide.

Conclusion

The present results establish the existence of an augmented F1-histone phosphokinase activity coinciding with a discrete cell cycle stage, mitosis (M). Control experiments have shown that this high chromatin-associated phosphokinase activity also exists in nonarrested mitotic cells and in metaphase-arrested cells of at least one other cell type (Table II). This augmented activity was also observed in both Colcemid and vinblastine-arrested monolayer or suspension grown Chinese hamster cells. Taken together, these properties argue against a trivial basis for the observed phenomenon and allow the tentative assumption that mitotic cell F1 phosphokinase, whose activity is greatly augmented over that found in interphase (S-G2) cells, is of general occurrence in cultured animal cells. Extension of these observations to a broader range of cells, however, will be needed to confirm whether F1-histone phosphorylation at the G2-M boundary is a legitimate marker in the normal cell cycle.

Additional experiments have been presented to show that there is increased incorporation of radiophosphorus into F1 *in vivo* corresponding to the observed electrophoretic mobility shift of mitotic cell F1 histone. This mobility shift can be directly duplicated by *in vitro* phosphorylation with the chromatin-associated enzyme. These observations, coupled with a

high F1 substrate specificity (Table III) and chromatin localization, have suggested that this chromatin enzyme is responsible for the *in vivo* mitotic cell F1 phosphorylation observed in earlier experiments (Lake *et al.*, 1972) and Figure 5.

The finding of cycle dependent differences in F1-phosphokinase activity in Chinese hamster cells is in apparent contradiction to two previous reports concerning phosphokinase activity in animal cells. Shepherd *et al.* (1971b) demonstrated a constant specific activity for 0.15 M NaCl-soluble phosphokinase(s) in Chinese hamster ovary cells synchronized by the isoleucine-depletion method. But, because cell doubling (progress of individual cells through a ~30-min mitosis) is extended over a 12- to 14-hr period in this system, detection of augmented activity contributed by mitotic cells would not be expected. As is seen in Figure 9 augmented activity is not detected until significant numbers of M cells are collected by vinblastine. Shepherd *et al.* (1971b) also reported a 1.76-fold stimulation by cAMP with F1 as substrate. This is seemingly at variance with the present data in Table I which show no cAMP stimulation in even the soluble activity of cytosol. In interpreting this result it is important to realize that the pH optimum for catalytic activity (~pH 9.0 for the chromatin bound phosphokinase activity) is not necessarily the pH optimum for demonstrating cAMP stimulation (Corbin *et al.*, 1972). In three experiments of the type shown in Table I we have been able to demonstrate a 1.9-fold cAMP stimulation of cytosol phosphokinase(s) by assay at pH 7.4. At no pH between 7.0 and 9.5, on the other hand, is the chromatin-bound activity stimulated by cAMP (Figure 2). This is the present basis for considering the phosphokinase activity of metaphase cell chromatin to be cAMP independent.

A second apparent inconsistency with the present results is a report by Balhorn *et al.* (1972b) that ^{32}P incorporation into F1-histone of 5- to 8-hr Colcemid-arrested HTC mitotic cells is minimal compared to cells exhibiting DNA synthesis. With no knowledge of phosphate (ATP) pool sizes or uptake rates over either Chinese hamster or HTC cell cycles, we can only comment from the experiments of Figure 9 and 10 that cells arrested longer than a few hours no longer exhibit maximal enzyme activity and would not be expected to maximally incorporate ^{32}P into F1 after an extended arrest time. Enhanced phosphokinase activity and F1 phosphorylation in mitotic cells as demonstrated in the present experiments are not in contradiction with previous observations that histone phosphorylation occurs during S phase; all our experiments show substantial enzyme activity and phosphorylation in interphase cells. Present concern is in explaining the differential in phosphokinase activity of M and S-G2 cells.

Three possible modes by which F1-phosphokinase(s) can reach high specific activity in mitotic cells are being considered: (1) translocation from other cell fractions to chromatin, (2) accumulation of enzyme to a higher steady-state level by increased net synthesis or slower degradation, and (3) activation of existing enzyme by interaction with small molecules (cAMP) and/or attendant protein modulators.

Translocation of cytoplasmic phosphokinase(s) to chromatin at mitosis seems excluded since F1-phosphokinase activity is elevated to various degrees in the total cell homogenate and in all mitotic cell fractions (Table I). Translocation *in vivo* would result in a corresponding decreased specific activity in extranuclear fractions. The possibility of translocation of phosphokinase during chromatin isolation at low ionic strength is not excluded by the present data. Even so, such cross contamination would not explain the elevated specific activity in all M cell fractions.

F1 phosphokinase could be accumulated as a peak or exponential enzyme (Mitchison, 1969) by increased net synthesis in G2. Because there is a natural decay of augmented F1-phosphokinase activity in M cells it is not possible to directly compare degradation rates in M *vs.* I cells in the presence of cycloheximide. Such a comparison would be necessary to exclude slower degradation rate as explaining augmented activity. In this regard it is noted that the natural decay of activity in mitotic cells is temporally similar to the decay in cycloheximide-treated interphase cells. A need for continued protein synthesis to maintain phosphokinase activity in chromatin (Table V) raises the possibility that the decay in mitotic cells is due to continued depression of protein synthesis in arrested-metaphase cells (Konrad, 1963; Johnston and Holland, 1965).

The third, and least clear, possibility that existing phosphokinase is regulated by cAMP gains some support from the present observation that chromatin-associated enzyme is cAMP independent and thus already maximally stimulated. However, involvement of cAMP is inconsistent with our inability to demonstrate stimulation of interphase phosphokinase to an equivalent metaphase cell level in any cell fraction (Table I). In actuality, it may be that all three modes considered here are instrumental in causing high phosphokinase activity in chromatin of mitotic cells.

In conclusion, the overall implication from our findings is that F1 phosphorylation is not a direct correlate of DNA synthesis as suggested previously (Ord and Stocken, 1966; Stevely and Stocken, 1968; Balhorn *et al.*, 1972a,b), since nuclear DNA synthesis does not occur during mitosis. Rather, a biological function for F1 phosphorylation consistent with its observed properties and intracyclic time of appearance would be that it is related to one of the unique metabolic or structural changes found in mitotic cells. These unique changes include chromosome condensation, organelle transitions (Robbins and Gonatas, 1964), surface changes (Fox *et al.*, 1971), and depressed gene activity (Farber *et al.*, 1972). This same event is pictured to occur to a lesser extent during traverse of interphase and not at all in G_0 cells. Further comparative examination of mitotic cells should eventually specify this event.

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Dissociation of *Escherichia coli* Ribosomes. Role of Initiation Factors†

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ABSTRACT: The protein which promotes dissociation of *Escherichia coli* 70S ribosomes has been purified and identified as the initiation factor 3 (IF-3). The dissociating activity of this factor (dissociation factor, DF) is stimulated by the addition of initiation factor 1 (IF-1) and GTP. The DF activity is also enhanced by crude initiation factor 2 (IF-2) although it is not entirely certain whether the active component is the same as IF-2. The addition of fMet-tRNA to ribosomes maximally dissociated by DF in the presence of IF-1, IF-2, GTP, and poly(A-U-G) leads to the association of subunits and to the formation of the 70S initiation complex. Thus, the requirements for the maximum dissociation of 70S ribosomes and the formation of the 70S initiation complex appear to be similar.

A protein factor which dissociates ribosomes was first reported in *Escherichia* coli (Subramanian *et al.*, 1968) and subsequently in *Bacillus stearothermophilus* (Bade *et al.*, 1969), *Saccharomyces cerevisiae* (Pêtre, 1970), rat liver (Lawford *et al.*, 1971), and rabbit reticulocytes (Lubsen and Davis, 1972). Recently, several groups have reported that the *E. coli* dissociation factor (DF) is the same as the initiation factor 3 (IF-3) (Albrecht *et al.*, 1970; Sabol *et al.*, 1970; Subramanian and Davis, 1970; Dubnoff and Maitra, 1971; Sabol and Ochoa, 1971; Grunberg-Manago *et al.*, 1971; also see Davis, 1971). However, there is a lack of agreement on certain other points such as the stimulation of DF activity by GTP and stoichiometry between DF molecules and ribosomes dissociated. It is therefore uncertain whether the dissociating activity of IF-3 is an integral part of its role in the initiation of protein synthesis or merely a side effect *in vitro*. Moreover, we have previously presented evidence that much of the DF activity is associated with a protein fraction enriched with IF-1 (Miall *et al.*, 1970).

In order to explain these discrepancies, we have further studied the involvement of initiation factors in the dissociation of ribosomes. We report in this paper that the DF activity is in fact associated with the IF-3 fraction, but that its activity is stimulated by the addition of IF-1, IF-2, and GTP. In agreement with these findings Noll and Noll (1972) have recently reported that the dissociating activity of IF-3 is enhanced by IF-1.

Materials and Methods

Preparation of 70S Ribosomes. Ribosomes were prepared from *E. coli* B cells, harvested in mid-log phase after slow cooling, washed once with 0.5 M NH_4Cl and once with 1.0 M NH_4Cl, and resuspended in 10 mM Tris-HCl (pH 7.4), containing 5 mM Mg^{2+}, 50 mM KCl, and 6 mM β-mercaptoethanol.

Preparation of DF and Initiation Factors. E. coli ribosomes were washed once with 0.1 M NH_4Cl and then extracted with 1.0 M NH_4Cl. The 1.0 M NH_4Cl extract was fractionated by

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