Phosphorylation of nuclear and DNA-binding proteins in proliferating and quiescent mammalian cells

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The dependence of cell proliferation on nuclear protein phosphorylation was studied with exponential-phase and stationary-phase cultures of Chinese-hamster ovary cells. Nuclear proteins were fractionated, according to their DNA-binding affinities, by using sequential extractions of isolated nuclei with increasing concentrations of NaCl. When viable whole cells were labelled with $H_3^{32}PO_4$, phosphorylation of nuclear proteins was found to be lower in quiescent cells than in proliferating cells. Phosphorylation of nuclear proteins soluble in 0.30 M-NaCl (less than 50% of these proteins bind to DNA) was greater than for those proteins soluble in higher salt concentrations (80–100% of these proteins bind to DNA). Cyclic AMP enhanced the phosphorylation of nuclear proteins soluble in 0.3 M-NaCl by 40–50%, and this stimulation was independent of cell growth. Cyclic AMP also increased the phosphorylation of nuclear proteins soluble in 0.6 M-NaCl and 2.0 M-NaCl by 40–50% in exponential-phase cultures, but not in stationary-phase cultures. Several examples of specific phosphorylation in response to cyclic AMP were observed, including a 35 000-mol.wt. protein in the 0.30 M-NaCl-soluble fraction and several proteins larger than 100 000 molecular weight within this fraction. A major peptide of molecular weight approx. 31 000 extracted with 0.6 M-NaCl was also phosphorylated. Its phosphorylation was independent of cyclic AMP in exponential-phase cultures, and it was not phosphorylated in plateau-phase cells. These changes in cell-growth-dependent phosphorylation occurred in the absence of any apparent qualitative changes in the nuclear protein molecular-weight distributions. These data demonstrate that (1) phosphorylation of nuclear proteins is dependent on the culture's proliferative status, (2) both cyclic AMP-dependent and cyclic AMP-independent specific phosphorylation occurs, and (3) the cyclic AMP-dependent growth-independent phosphorylation that occurs does not appear to be a modification of DNA-binding proteins, whereas the cyclic AMP-dependent growth-dependent phosphorylation does involve modification of DNA binding proteins.

Phosphorylation of nuclear proteins has been suggested to be an important mechanism of genetic regulation in eukaryotic cells (for review see Kleinsmith, 1975), although the evidence is circumstantial and not conclusive (Stein *et al.*, 1978). Other work has pointed to a possible role for phosphorylation of nuclear proteins, especially histones, in determining chromatin structure (Louie & Dixon,

1972; Tobey *et al.*, 1974). It is thought that histone phosphorylation may only indirectly, if at all, affect gene transcription.

Evidence supporting this hypothesis includes the findings that histone phosphorylation is cell-cycle-specific (Gurley *et al.*, 1974), that phosphorylation of histone H1 occurs only in proliferating tissues (Balhorn *et al.*, 1972), and not in quiescent but genetically active tissues (Balhorn *et al.*, 1971).

In contrast with the histones, the non-histone nuclear proteins are phosphorylated in both proliferating and non-proliferating tissues (Ganpath *et al.*, 1977). Moreover, phosphorylation of the non-histone nuclear proteins affects gene trans-

Abbreviations used: CHO cells, Chinese-hamster ovary cells; SDS, sodium dodecyl sulphate.

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cription in reconstituted chromatin (Kleinsmith *et al.*, 1976). Further, tumour-specific (Ganpath *et al.*, 1977), tumour-regression-associated (Cho-Chung *et al.*, 1978), hormone-dependent (Bustos *et al.*, 1977) and cyclic nucleotide-dependent (Johnson, 1977) phosphorylation of non-histone nuclear proteins has been reported. In the most widely studied system, rat liver, Johnson & Allfrey (1972) have shown that cyclic AMP stimulates the phosphorylation of a small number of molecular-weight groups of the material they called nuclear acidic proteins. Through the work of Kuo & Greengard (1969) and Langan (1969), the action of cyclic AMP is thought to involve the activation of cyclic AMP-dependent protein kinase(s), which phosphorylate nuclear proteins such as histones and non-histone chromatin proteins.

Because of our interest in understanding the possible role(s) of the non-histone nuclear proteins in the regulation of cell proliferation, and because of the possible importance of phosphorylation of these proteins, this laboratory has been investigating the expression of the cyclic AMP-dependent and -independent protein kinases in synchronous proliferating and quiescent cells (Costa *et al.*, 1975, 1976, 1977, 1978). Changes in intracellular cyclic AMP concentration correlated closely with changes in the activation of the cyclic AMP-dependent protein kinases (Costa *et al.*, 1975, 1976, 1978). These results were consistent with the original hypothesis that all cellular effects of cyclic AMP were mediated via the activation of cyclic AMP-dependent protein kinases (Kuo & Greengard, 1969). Using Chinese-hamster ovary (CHO) cells as a model system, we found that the cyclic AMP-dependent protein kinases, which were evident as two distinct forms on DEAE-cellulose chromatography (Costa *et al.*, 1976), were differentially expressed throughout the cell cycle. Further, quiescent cultures were nearly deficient in the type II enzyme, whereas this form is expressed as the predominant species in cells at the G₁/S-phase boundary (Costa *et al.*, 1978). Also, the cyclic AMP-dependent enzymes were predominantly cytoplasmic in origin, whereas the nuclear enzymes, of which at least four forms were measurable on DEAE-cellulose chromatography, were cyclic AMP-independent (Costa *et al.*, 1977).

The experiments reported in the present paper were undertaken in order to correlate changes in cyclic AMP-dependent and -independent phosphorylation of nuclear proteins with previously reported changes in cyclic AMP concentrations and cyclic AMP-dependent and -independent protein kinase activities in proliferating and quiescent CHO cells. The results obtained demonstrate significant dependence of phosphorylation of nuclear protein on cell proliferation. Cyclic AMP-mediated phos-

phorylation of protein fractions displaying low affinity for DNA is independent of cell proliferation, whereas similar phosphorylation of protein fractions with high affinity for DNA is dependent on cell division.

Experimental

Materials

Foetal bovine sera, McCoy's 5A medium, Puck's saline A and antibiotic/antimycotic solutions were ordered from GIBCO (Grand Island, NY, U.S.A.). H₃³²PO₄, [γ-³²P]ATP, [³H]leucine (15 Ci/mmol) and [*Me*-³H]uridine (20 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Dibutyryl cyclic AMP (grade A), sodium butyrate and theophylline were purchased from Calbiochem (Los Angeles, CA, U.S.A.). Histone (calf thymus mixture) was also purchased from Calbiochem. SDS (electrophoresis grade) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³H]Tryptophan was purchased from Schwarz-Mann (Orangeburg, NY, U.S.A.).

Cell-culture techniques

CHO cells, having a doubling time of 13–15 h, were routinely maintained in McCoy's 5A medium, in monolayer cultures at 37°C in a CO₂/air (1:19) atmosphere. The medium was supplemented with 20% (v/v) foetal bovine serum, 100 units of penicillin/ml, 100 µg of streptomycin/ml and 0.25 µg of Fungizone/ml. In order to obtain large cell numbers for nuclear isolation, cells were grown in roller bottle cultures (1500 cm² surface area). After cells had been seeded into the bottles, each of which contained 150 ml of medium, the cultures were gassed briefly with CO₂ and placed in a 37°C room on a roller mill. Cultures were seeded at a density of 2 × 10⁴ cells/cm². Exponential-phase cultures were obtained 48 h later. Plateau-phase cells were defined as 5-day-old cultures. Viability, labelling indices and mitotic indices of cultures grown under these conditions have been previously reported (Gerner *et al.*, 1976). In selected experiments, cultures were labelled for 48–120 h with 0.25 µCi of [³H]leucine/ml in medium adjusted to contain 10% of the normal unlabelled leucine concentration. The results (not shown) confirmed that cell growth rate and viability were unaffected under these conditions. Measurements of phosphorylation in intact cells were accomplished by labelling whole cells in medium, modified to exclude unlabelled inorganic phosphate, containing 50 µCi of H₃³²PO₄/ml. The radiolabelled phosphate was added for a total of 120 min. Then 30 min after addition of the H₃³²PO₄ (a period to allow uniform labelling of the ATP pool), either saline or 500 µM-dibutyryl cyclic AMP was added to the cultures and incubation proceeded for the final

minutes. This concentration of dibutyl cyclic AMP was chosen because it completely activates intracellular cyclic AMP-dependent protein kinases (results not shown). The labelling periods were stopped by removing the culture medium and washing once with cold Puck's saline A. Cells were immediately scraped, by using a rubber 'police-man', into cold Puck's saline A and placed in an ice bath.

Preparation of nuclear and cytosol fractions

Nuclei were isolated from cultures by using a modification of the Triton X-100 extraction (with 80mM-NaCl, 10mM-Tris/HCl buffer, pH 7.0, 1mM-phenylmethanesulphonyl fluoride, 20mM-EDTA and 10mM-NaF) procedure as previously described (Gerner & Humphrey, 1973; Costa *et al.*, 1977). These nuclei were then extracted sequentially with buffer (10mM-Tris/HCl buffer, pH 7.0, containing 1mM-phenylmethanesulphonyl fluoride and 10mM-NaF) containing 0.15M-, 0.30M-, 0.60M- and 2.0M-NaCl. The extraction procedure consisted of homogenizing the nuclear pellet in the salt buffers by using 20 strokes of a hand-operated Teflon-pestle homogenizer. Total extraction time was 10min plus the centrifugation period. Extraction was judged to be complete for each salt concentration, as longer or additional extractions did not solubilize additional significant protein amounts.

DNA-cellulose chromatography

Native and denatured DNA-cellulose were prepared by the methods of Litman (1968) and Alberts & Herrick (1971) as described elsewhere (Magun & Dorsett, 1977). Both forms of DNA-cellulose contained 2mg of calf thymus DNA (Worthington Biochemical Corp., Freehold, NJ, U.S.A.)/ml of column volume. The ratio of protein applied to DNA in the column was 5:1 for all experiments reported here. Proteins were first applied to native and then to denatured DNA-cellulose columns. Columns were washed extensively (6 column volumes) with buffer A (50mM-NaCl/0.1% Brij 58/1mM-phenylmethanesulphonyl fluoride/1mM-EDTA/10mM-2-mercaptoethanol/10% (v/v) glycerol/10mM-Tris/HCl buffer, pH 8.1, to remove proteins without affinity for DNA. Proteins remaining on the columns were then eluted with either 0.25M- or 2.0M-NaCl in buffer A for the nuclear proteins or NaCl concentrations ranging from 0.1 to 2.0M in buffer A for cytosol fractions. Recovery of proteins applied to the DNA-cellulose columns was essentially 100%.

Phosphorylation of nuclear protein extracts in vitro

Nuclear proteins were extracted from unlabelled CHO cells. The extracts were phosphorylated by incubation for 1 min at 30°C in medium containing

[γ - 32 P]ATP (5 μ M; about 1×10^5 c.p.m./pmol), 10mM-MgCl₂, 50mM-phosphate buffer, pH 6.5, 5mM-aminophylline and 5mM-NaF in the absence and in the presence of both 10 μ M-cyclic AMP and a purified cyclic AMP-dependent protein kinase. The cyclic AMP-dependent protein kinase employed was isolated from bovine heart by using a previously published procedure (Kuo & Greengard, 1972). Approx. 50–70 μ g of nuclear protein was used for the procedures for phosphorylation *in vitro*. The nuclear protein extracts used for these procedures were dialysed against 50mM-phosphate buffer, pH 6.5, containing 0.1mM-EDTA in order to remove the NaCl. The reaction was initiated by addition of the [γ - 32 P]ATP and terminated by adjusting the samples to 1% SDS, 2% (v/v) β -mercaptoethanol, 20mM-EDTA and 10% (v/v) glycerol. A 50 μ g sample of protein from this reaction was applied to polyacrylamide gels and electrophoresis was performed as described below.

Polyacrylamide-gel electrophoresis

Protein samples were prepared for SDS/polyacrylamide-gel electrophoresis by adjusting them to 1% SDS, treating in boiling water for 1 min and then dialysing the samples against the electrophoresis sample buffer (0.1% SDS/1% 2-mercaptoethanol/10mM-phosphate buffer, pH 7.2) for 48 h. The SDS/polyacrylamide-gel electrophoresis method described by Laemmli (1970) was used. The separation gel included 8.75% acrylamide and 0.23% methylenebisacrylamide. Separation gels were routinely poured to be 12.5 cm in length with a 0.5 cm stacking gel. Molecular weights were determined by using a commercially available kit (Bio-Rad) including myosin, galactosidase, phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase and soya-bean trypsin inhibitor. The gels were stained with Coomassie Blue R-250 in 10% (v/v) acetic acid and 50% (v/v) methanol containing 0.05% Coomassie Blue and destained electrophoretically with a 7.5% acetic acid/5% methanol mixture. After this staining and destaining procedure, some gels were treated with hot 5% (w/v) trichloroacetic acid (90°C for 30 min). This was followed by extensive washing with 5% trichloroacetic acid at room temperature (two separate washings, 2 h each with stirring, 1 litre of trichloroacetic acid being used for about eight gels) (Bhorjee & Pederson, 1976). All gels having visible protein bands were sliced into 1.0 mm segments and the radioactivities were counted in a toluene/NCS/Omnifluor mixture (Gerner & Humphrey, 1973). In selected experiments, proteins solubilized from nuclei were directly extracted with phenol to remove nucleic acids before dialysis against appropriate buffers.

Measurement of protein kinase activity

Protein kinase activity was measured as previously described (Costa *et al.*, 1975, 1976, 1978) and modified (Costa *et al.*, 1977). No exogenous substrate was used. The various salt extracts were dialysed against 5 mM-aminophylline/1 mM-EDTA/10 mM-phosphate buffer, pH 6.5. This extract was centrifuged at 50000g for 20 min and the resulting supernatant was taken for assay of protein kinase activity. When exogenous purified kinase was used, the procedure was identical with that described above with the exception of the addition of the purified kinase.

Measurement of DNA content per cell

In order to determine the cell-cycle distribution of cells in the exponential-phase and plateau-phase cultures, measurements of DNA content per cell were made by using flow cytometry techniques. A Coulter model TPS-1 fluorescent activated cell sorter (Coulter Electronics, Hialeah, FL, U.S.A.) was used in the non-sort mode. Cultures were harvested, washed with Puck's saline A, fixed with 70% ethanol, washed with Hanks balanced salt solution and then stained with propidium iodide (6.9 mM) in 3.8 mM-sodium citrate.

Determination of protein content

Protein concentrations were measured by using the procedure described by Bradford (1976), with bovine serum albumin as standard.

Results

Fig. 1 shows the cell age distributions, in terms of DNA content per cell, for the exponential-phase cultures (Fig. 1a) and plateau-phase cultures (Fig. 1b) grown as described in the Experimental section. It is clear that the plateau-phase cultures are deficient in cells with higher DNA contents compared with the exponential-phase cultures. The DNA distributions shown in Fig. 1(a) are consistent with labelling indices for exponential-phase cultures of approx. 50% and mitotic indices of about 5%. A crude area-under-the-curve analysis of Fig. 1(a) yields approx. 35% G₁-phase cells, 50% S-phase cells and 15% G₂-plus-M-phase cells (calculations not shown). Viability for both cultures is approx. 80–90%.

To characterize the nuclear proteins of CHO cells, isolated nuclei were sequentially extracted with buffers containing increasing concentrations of NaCl. Table 1 shows that, in exponential-phase

Fig. 1. DNA distributions of CHO-cell cultures

Experimental details are given in the text. Channel 21 corresponds to a $2n$ DNA content, and channel 42 corresponds to a $4n$ DNA content. (a) DNA distribution from exponential-phase cells; (b) DNA distribution of plateau-phase cultures. Labelling indices, mitotic indices and viability measurements for cultures as shown here have been previously reported (Gerner *et al.*, 1976).

Table 1. *Characteristics of nuclear protein fractions obtained by sequential salt extractions of nuclei isolated from exponential cultures*

CHO cells, growing exponentially, were exposed to [^3H]tryptophan (5 $\mu\text{Ci/ml}$, 15 Ci/mmol) or $\text{NaH}_2^{32}\text{PO}_4$ (50 $\mu\text{Ci/ml}$, >1000 Ci/mmol) for 2h periods. In the latter case the growth medium was modified to exclude unlabelled phosphate. After the labelling procedures, cultures were harvested and nuclei were isolated as described in the Experimental section. The nuclei were then sequentially treated with 0.15M-, 0.3M-, 0.6M- and 2.0M-NaCl to extract nuclear proteins, as discussed in the text. In this Table total nuclear protein refers to those soluble in up to 2.0M-NaCl. The values shown for protein yield represent the averages for eight individual experiments. The values for specific radioactivities are averages of two replicate experiments.

Concn. of NaCl (M) used for extraction	Protein yield		[^3H]Tryptophan sp. radioactivity (c.p.m./ μg of protein)	[^{32}P]Phosphate sp. radioactivity (c.p.m./ μg of protein)
	($\mu\text{g}/10^7$ cells)	(% of total)		
0.15	63	22	421	302
0.30	86	30	469	264
0.60	104	36	338	109
2.0	35	12	141	107

CHO cells, most of the nuclear proteins are solubilized with NaCl concentrations between 0.3 and 0.6M. Also shown in Table 1 is the [^3H]tryptophan specific radioactivity for protein solubilized by the stated NaCl concentrations. The data demonstrate that the values (1) are highest for proteins soluble in 0.15M- and 3.0M-NaCl, (2) decrease for protein soluble in 0.6M- and 2.0M-NaCl and (3) do not decrease to zero with increasing salt concentration. This latter point indicates that the proteins solubilized by the higher salt concentrations are not exclusively histones but also include non-histone proteins. As with [^3H]tryptophan specific radioactivity, the [^{32}P]phosphate specific radioactivity is highest for those proteins solubilized by the lower NaCl concentration. The [^{32}P]phosphate specific radioactivity decreases for proteins soluble in up to 0.6M-NaCl and then remains constant for proteins soluble in 2.0M-NaCl.

Fig. 2 shows the molecular-weight distributions on SDS/polyacrylamide-gel electrophoresis of proteins from nuclei of exponential-phase and quiescent cultures. These nuclei were extracted with a range of salt concentrations from 0.15M-NaCl up to 2.0M-NaCl. The results demonstrate that there are few qualitative differences between exponential-phase or plateau-phase cultures in molecular-weight distributions of polypeptides isolated in this manner. There are quantitative differences evident for most of the fractions. Solubilization of proteins from isolated nuclei with each salt concentration was essentially complete under the conditions reported, since re-extraction did not elute any additional significant amounts of protein (results not shown).

It was decided to investigate the location of endogenous protein kinase activity and endogenous substrate activity for cyclic AMP-dependent and -independent protein kinases in the various nuclear protein fractions. The results given in Table 2 show that no endogenous protein kinase activity is

measurable in nuclear proteins insoluble in 0.60M-NaCl. In nuclear proteins soluble in 0.60M-NaCl there is little or no cyclic AMP-stimulatable activity measurable with the exception of the 0.15M-NaCl-soluble proteins. In addition, the protein kinase activity measured in the presence of exogenous cyclic AMP in the 0.3M-NaCl-soluble fraction is less than that measured in its absence, suggesting the possibility of cyclic AMP-dependent phosphatase activity in this fraction. Also presented in Table 2 is the endogenous substrate activity of nuclear proteins. All nuclear protein fractions show some substrate activity for cyclic AMP-dependent protein kinases *in vitro*. Those nuclear proteins insoluble in 0.6M-NaCl show no substrate activity in the absence of exogenous cyclic AMP in the reaction mix, but all fractions show substrate activity *in vitro* in the presence of cyclic AMP.

Because of the necessity to use dibutyryl cyclic AMP to elevate intracellular cyclic AMP concentrations in intact cells, control studies were done to ensure that the butyrate was not responsible for possible changes in nuclear protein phosphorylation patterns. One set of these controls is shown in Table 3, which gives the results of nuclear protein phosphorylation performed with saline, dibutyryl cyclic AMP, dibutyryl cyclic AMP plus the phosphodiesterase inhibitor theophylline, theophylline alone, n-butyrate alone or n-butyrate plus theophylline as the stimulating agents. In Table 3 only the results for those proteins soluble in 0.3M-NaCl are shown. However, all nuclear protein fractions were checked and the findings were similar to those shown here. It is clear that dibutyryl cyclic AMP leads to approximately a 50% increase in the [^{32}P]phosphate incorporation measured after phenol extraction to remove contaminating nucleic acid. The presence of a phosphodiesterase inhibitor is not necessary when 500 μM -dibutyryl cyclic AMP is used. Theophylline by itself does result in an

Fig. 2. SDS/polyacrylamide-gel electrophoretograms, stained with Coomassie Blue, showing the molecular-weight distributions of proteins extracted from nuclei of exponential-phase (a, c, e and g) or plateau-phase (b, d, f and h) cultures

Experimental details are given in the text. Gels show those proteins solubilized from nuclei by sequential extraction with 0.15M-NaCl (a and b), 0.3M-NaCl (c and d), 0.60M-NaCl (e and f) and 2.0M-NaCl (g and h) buffered solutions.

Fig. 3. [^3H]Leucine (●) and [^{32}P]phosphate (○) distribution and $^3\text{H}/^{32}\text{P}$ ratios (■) on SDS/polyacrylamide-gel electrophoretograms of nuclear proteins obtained by sequential salt extraction

Exponential-phase cultures were labelled with the radioisotopes as described in the Experimental section. After the labelling periods, cultures were harvested by scraping cells into cold Puck's saline A, nuclei were isolated and nuclear protein fractions were obtained. (a) and (b) represent nuclear proteins soluble in 0.15M-NaCl, (c) and (d) represent those soluble in 0.3M-NaCl, and (e) and (f) represent those soluble in 0.60M-NaCl. (a), (c) and (e) show results from cultures labelled in the absence of dibutyryl cyclic AMP, and (b), (d) and (f) show results from cultures labelled in the presence of 500 μM -dibutyryl cyclic AMP. The gels shown are representative examples. The Coomassie Blue stain distributions that correspond to these gels are shown in Fig. 2.

Table 2. Substrate specificity for cyclic AMP-dependent protein kinase and endogenous kinase activity of nuclear proteins fractionated by NaCl solubility

Nuclei were isolated from exponentially growing CHO cells as described in the Experimental section. Nuclear proteins were then fractionated by using sequential extraction procedures in buffer of increasing salt concentrations. The endogenous substrate specificity of those proteins for protein kinases was then measured *in vitro* by using exogenous cyclic AMP-dependent protein kinase purified from bovine heart as described by Kuo & Greengard (1972). The endogenous protein kinase activity of the nuclear protein fractions isolated by sequential salt extractions was determined, again *in vitro*, by using endogenous substrates as described in detail in the Experimental section. The values shown represent the averages of triplicate determinations in a representative experiment. Three replicate experiments were performed.

Fraction [concn. of NaCl (M) used for extraction]	Reaction conditions	Protein kinase activity (pmol of ³² P incorporated/min per mg of protein)	
		–Cyclic AMP	+Cyclic AMP
0.15	Exogenous kinase added to reaction mix	254.4	718.2
0.30		327.8	579.0
0.60		139.7	925.0
1.00		0	0
4.00		0	0
0.15	No exogenous kinase to reaction mix	219.7	307.7
0.30		363.3	87.0
0.60		106.3	114.4
1.0		0	0
4.0		0	0

Table 3. Effects of various additions on the phosphorylation of nuclear proteins soluble in 0.3 M-NaCl

Whole cells were labelled for 2 h with NaH₂³²PO₄ in growth medium minus unlabelled phosphate. During the labelling period dibutyl cyclic AMP (0.5 mM), theophylline (0.5 mM) and n-butyrate (1.0 mM), alone or in various combinations as indicated, were added to the medium. After the 2 h interval, the culture medium was decanted and unlabelled medium was immediately added to stop incorporation. Cultures were then harvested, nuclei were isolated and nuclear protein fractions were obtained by using the sequential salt extraction procedure as described in the Experimental section. Proteins were treated with phenol to remove contaminating nucleic acids. Portions of the final samples were then taken for both counting of ³²P radioactivity incorporated and for protein determinations by the Bradford (1976) technique in order to obtain specific-radioactivity values. The data shown are averages for triplicate determinations in a representative experiment in a series of three replicates.

Additions	Specific radioactivity			
	Before phenol (³² P c.p.m./μg of protein)	After phenol (³² P c.p.m./μg of protein)	% phenol-soluble	% of control after phenol
None (control)	1177	264	78	100
Dibutyl cyclic AMP	1892	394	79	149
Dibutyl cyclic AMP + theophylline	1400	392	72	148
Theophylline	1446	331	77	125
n-Butyrate	1299	261	80	99
n-Butyrate + theophylline	1117	317	72	120

Fig. 4. [³H]Leucine (●) and [³²P]phosphate (○) distributions and ³²P/³H ratios (■) on SDS/polyacrylamide-gel electrophoretograms of proteins from plateau-phase conditions

Experimental details are given in the text. (a) and (b) represent nuclear proteins soluble in 0.15 M-NaCl, (c) and (d) represent nuclear proteins soluble in 0.30 M-NaCl, and (e) and (f) represent nuclear proteins soluble in 0.60 M-NaCl. As in Fig. 3, (a), (c) and (e) show results from cultures labelled in the absence of dibutyl cyclic AMP, and (b), (d) and (f) show results from cultures labelled in the presence of 500 μM-dibutyl cyclic AMP. Fig. 2 shows the Coomassie Blue stain profiles for these gels.

Table 4. *Comparison of relative phosphorylation of nuclear proteins from exponential-phase and plateau-phase cells in response to cyclic AMP*

Cultures were in either exponential phase or plateau phase of growth and had previously been labelled for 2 h with $\text{NaH}_2^{32}\text{PO}_4$. This was done both with and without the addition of dibutyryl cyclic AMP to the labelling medium. Cultures were then harvested, nuclei were obtained and nuclear proteins were fractionated by using the sequential salt-extraction procedure as described in the Experimental section. The protein fractions were then dialysed against buffer and run on SDS/polyacrylamide gels. The gels were stained with Coomassie Blue for detection of protein bands. The gels were treated with hot trichloroacetic acid as detailed in the Experimental section to remove contaminating nucleic acids, and then fractionated into 1 mm segments for measurement of ^{32}P radioactivity by using the liquid-scintillation counting techniques indicated in the Experimental section. The values shown are corrected for ^{32}P decay, and the protein values were determined from known [^3H]leucine specific radioactivities. The values shown are from a representative experiment in a series of four replicates.

Growth state	Fraction [concn. of NaCl (M) used for extraction]	Specific radioactivity (^{32}P c.p.m./ μg of protein)		Ratio of specific radioactivities (+dibutyryl cyclic AMP/–dibutyryl cyclic AMP)
		–Dibutyryl cyclic AMP	+Dibutyryl cyclic AMP	
Exponential phase	0.15	354	498	1.41
	0.30	711	1002	1.41
	0.60	413	598	1.45
	2.0	337	512	1.52
Plateau phase	0.15	310	452	1.46
	0.30	531	813	1.53
	0.60	340	330	0.97
	2.0	208	228	1.10

increase in [^{32}P]phosphate incorporation. n-Butyrate caused essentially no change in phosphorylation over the saline control. Not shown is a comparison of the Coomassie Blue-detected molecular-weight distributions on SDS/polyacrylamide gels from all of these fractions. The specific radioactivity of these fractions is unchanged by butyrate, and the molecular-weight distributions of polypeptides in all fractions were unaffected. It should be noted that our molecular-weight distribution only spans the range from 200 000 to 20 000. Also, it should be noted that approx. 70–80% of the ^{32}P radioactivity isolated in the nuclear protein fraction was phenol-soluble.

Figs. 3 and 4 show the results from phosphorylation of intact cells in exponential-phase cultures (Fig. 3) and in plateau-phase cultures (Fig. 4). Conditions of phosphorylation were identical. The results represented show [^3H]leucine radioactivities (c.p.m.), which are a measure of total protein, [^{32}P]phosphate incorporation after 2 h labelling, $^{32}\text{P}/^3\text{H}$ ratios and molecular-weight distributions. Only the gels for those proteins soluble in 0.15 M-, 0.3 M- and 0.6 M-NaCl are shown; the results for those proteins soluble in 2.0 M-NaCl are summarized in Table 4. The results demonstrate that, in most cases, cultures phosphorylated in the presence of exogenous cyclic AMP show general increases in [^{32}P]phosphate incorporation. There are specific examples of peptides that show greatly

enhanced phosphorylation in response to cyclic AMP. These include peptides of molecular weight above 100 000 in the 0.3 M-NaCl-soluble nuclear proteins of exponential-phase cultures (Fig. 3) (fractions 0–30) and in a specific polypeptide that occurs at fraction no. 84 in Fig. 3(d). It is noteworthy that some major polypeptides do not show enhanced phosphorylation in response to dibutyryl cyclic AMP treatment. In Figs. 3(e) and 3(f) a major polypeptide, which occurs at fractions 95–100, is phosphorylated to the same degree independent of whether dibutyryl cyclic AMP was present during the labelling period or not.

Results shown in Fig. 4 describe the findings from phosphorylation of plateau-phase cells. In those peptides soluble in 0.15 M- and 0.3 M-NaCl, there is a general enhancement in the [^{32}P]phosphate incorporation into nuclear proteins. There is specific phosphorylation observed in high-molecular-weight polypeptides (fractions 0–40) and in the polypeptide in the 0.3 M-NaCl-soluble fraction that appears at approximately fraction no. 90. In contrast with the exponential-phase-culture results, the 0.6 M-NaCl-soluble fraction from plateau-phase cultures does not show enhanced phosphorylation (increased $^{32}\text{P}/^3\text{H}$ ratios). In addition, the peptide appearing at fractions 95–100 in the 0.6 M-NaCl-soluble fraction does not seem to be phosphorylated in plateau-phase cultures, either in the absence or in the presence of dibutyryl cyclic AMP.

The results from Figs. 3 and 4 have been simplified and summarized in Table 4. Similarly to the results in Table 3, Table 4 shows that for the 0.3M-NaCl-soluble nuclear proteins phosphorylated in intact cells dibutyryl cyclic AMP increases phosphorylation by 40–50%. It should be noted that there are quantitative differences in the relative degree of phosphorylation of these fractions. The 0.3M-NaCl-soluble nuclear proteins showed the highest degree of phosphorylation, whereas the 0.15M-NaCl- and 2.0M-NaCl-soluble fractions show the least amount of phosphorylation, both cyclic AMP-dependent and -independent.

Similarly to exponential-phase cultures, plateau-phase nuclear proteins soluble in 0.3M-NaCl are increased 40–50% in their phosphorylation. In contrast with exponential-phase cells, the proteins insoluble in 0.3M-NaCl show no stimulated phosphorylation in response to dibutyryl cyclic AMP. This is shown in more detail in Figs. 4(e) and 4(f). Evident in Table 4 is the fact that plateau-phase cultures in general show a lower degree of phosphorylation when expressed as a specific radioactivity.

In order to determine their affinity for native or denatured forms of DNA, nuclear proteins, obtained from exponential-phase cultures, were applied sequentially to native and denatured DNA–cellulose columns after removal of the contaminating DNA (see the Experimental section). Fig. 5 shows the amount of each fraction that binds to the double-stranded form of DNA. The ability of 0.25M-NaCl and of 2.0M-NaCl to displace those proteins is also shown. The fraction of nuclear proteins that binds to the double-stranded form of DNA increases as the concentration of NaCl used to solubilize those nuclear proteins increases. Most of the DNA-binding proteins are eluted by 0.25M-NaCl for each nuclear protein fraction except the 2.0M-NaCl nuclear protein extract. This latter fraction is removed from double-stranded DNA almost exclusively by 2.0M-NaCl. Only a small portion of the nuclear proteins shows an affinity for single-stranded DNA. In contrast with the nuclear proteins binding to double-stranded DNA–cellulose, the nuclear proteins that bind to single-stranded DNA–cellulose did not show a relationship between the salt concentration necessary to remove them from nuclei and the salt concentration necessary to displace them from single-stranded DNA–cellulose. In both groups there are quantitatively more proteins binding weakly, or elutable at 0.25M-NaCl, than there are proteins binding tightly, or elutable at 2.0M-NaCl, for proteins solubilized by up to 0.6M-NaCl. There is a higher percentage of proteins solubilized in 2.0M-NaCl binding tightly to both forms of DNA.

Fig. 6 shows the SDS/polyacrylamide-gel electrophoretograms of the polypeptides, displaying affinity

Fig. 5. Percentage of proteins binding either double-stranded DNA (a) or single-stranded DNA (b)

Experimental details are given in the text. ●, Percentages of those proteins binding either form of DNA that are eluted at low salt concentrations (0.25M-NaCl); ○, percentages of those proteins that are eluted at high salt concentration (2.0M-NaCl). The abscissa shows, on a logarithmic scale, the molar salt concentration required to solubilize these proteins from nuclei, as in Table 1.

for double-stranded and single-stranded forms of DNA from nuclear fractions, solubilized by 0.3M-, 0.6M- and 2.0M-NaCl. Because of the very small percentage of proteins binding DNA from the 0.15M-NaCl-soluble fraction, these gels are not shown. The polypeptide profiles of DNA-binding proteins from all three fractions are different. Although the nuclear proteins displaying weak (0.25M-NaCl-elutable) or strong (2.0M-NaCl-elutable) affinities for native DNA are qualitatively different in the 0.3M-NaCl- and 0.6M-NaCl-soluble fractions, the nuclear proteins soluble in the 2.0M-NaCl fraction display only quantitative differences at most. Because of the small percentage of proteins displaying affinity for denatured DNA–cellulose, only the 0.3M-NaCl-soluble polypeptides binding

Table 5. *Effect of cyclic AMP-mediated phosphorylation on DNA-binding characteristics of 0.3M-NaCl-soluble nuclear proteins*

Cultures in exponential growth were first labelled with [^3H]leucine to label cellular proteins uniformly, and then labelled with $\text{NaH}_2^{32}\text{PO}_4$ both with and without the addition of dibutyryl cyclic AMP. Cultures were then harvested, nuclei were obtained and nuclear proteins were fractionated by using the sequential salt extraction procedure described in the Experimental section. The fraction soluble in 0.3M-NaCl was then treated with phenol to remove contaminating nucleic acids, dialysed against buffer and run on DNA-cellulose columns. The sequence was first native DNA-cellulose, with the material not interacting with this column then being applied to denatured DNA-cellulose. Both columns were then washed with first 0.25M-NaCl and then 2.0M-NaCl to elute proteins that remained on the columns. [^3H]Leucine radioactivity was then used as a measure of the amount of proteins binding to the various columns. The $^{32}\text{P}/^3\text{H}$ ratio was used to represent the relative phosphorylation per protein amount. The ratio of $^{32}\text{P}/^3\text{H}$ specific-radioactivity ratios in the presence and in the absence of dibutyryl cyclic AMP was used to assess the effect of dibutyryl cyclic AMP on the phosphorylation of proteins binding some form of DNA. The values shown are from a representative experiment in a series of three replicates.

DNA-cellulose column	Concn. of NaCl (M) used for elution	% of [^3H]leucine applied		$^{32}\text{P}/^3\text{H}$ specific-radioactivity ratio (after phenol extraction)		Ratio of $^{32}\text{P}/^3\text{H}$ specific-radioactivity ratios (+dibutyryl cyclic AMP/ -dibutyryl cyclic AMP)
		-Dibutyryl cyclic AMP	+Dibutyryl cyclic AMP	-Dibutyryl cyclic AMP	+Dibutyryl cyclic AMP	
Native	0.25	22.01	27.82	0.35	0.36	1.03
	2.0	17.22	15.42	0.42	0.35	0.83
Denatured	0.25	6.17	6.09	0.22	0.23	1.04
	2.0	3.57	4.66	0.41	0.39	0.95
Total bound to DNA		48.97	53.99			

weakly to single-stranded DNA and the 2.0M-NaCl-soluble polypeptides binding tightly to single-stranded DNA are shown in comparison with their protein counterparts binding to native DNA-cellulose. Qualitative differences are evident in the polypeptide distributions between proteins binding to denatured DNA-cellulose and those binding to native DNA-cellulose in these two fractions. In addition, only one polypeptide appearing at approx. 20 500 molecular weight is evident in the 2.0M-NaCl-soluble protein fraction binding to denatured DNA.

The final point of interest was to determine whether the nuclear proteins phosphorylated in response to dibutyryl cyclic AMP were DNA-binding proteins or whether they were nucleoplasmic proteins. In this series of experiments, intact cells were phosphorylated in the absence and in the presence of 500 μM -dibutyryl cyclic AMP in the culture media. Nuclei were purified and the 0.3M-NaCl-soluble nuclear proteins were extracted. This fraction was chosen since it contains a substantial portion of proteins that do not display DNA-binding activity. After the proteins had been isolated and dialysed against an appropriate buffer, they were applied sequentially to native and denatured DNA-cellulose columns. As shown in Table 5, the proteins phosphorylated in response to dibutyryl cyclic AMP do not appear to be DNA-binding proteins, since the $^{32}\text{P}/^3\text{H}$ ratio does not increase significantly in the

presence of dibutyryl cyclic AMP. In fact, a slight decrease is seen for proteins eluted by 2.0M-NaCl. A second point is that the relative phosphorylation, presented in the form of $^{32}\text{P}/^3\text{H}$ ratios, is 1.7–1.9-fold greater for proteins that bind more tightly (i.e. eluted at higher NaCl concentrations from the DNA-cellulose columns) to single-stranded DNA-cellulose columns. These changes occur in the absence of any measurable changes in the percentage of proteins that bind the denatured form of DNA-cellulose. Changes in the relative phosphorylation of native DNA-binding proteins with NaCl concentration required to elute them from columns were less significant.

Discussion

A great deal of circumstantial evidence exists to suggest a role for phosphorylation of non-histone nuclear proteins in cell regulation, although most investigators have interpreted the results as implying control of gene activation (for representative reviews see Kleinsmith, 1975; Johnson, 1977; Stein *et al.*, 1978). There exists an equally large body of evidence suggesting that histone phosphorylation is primarily associated with chromatin structural changes associated with cell proliferation (Balhorn *et al.*, 1971, 1972; Tobey *et al.*, 1974; Gurley *et al.*, 1974, 1978), rather than a specific modification

Fig. 6. *SDS/polyacrylamide-gel profiles of the polypeptides, binding to native and denatured DNA-cellulose, from nuclear protein fractions obtained by sequential salt extraction of nuclei*

Experimental details are given in the text. (a), (b) and (c) Proteins solubilized by 0.3 M-NaCl; (d) and (e) polypeptides solubilized by 0.6 M-NaCl; (f), (g) and (h) polypeptides solubilized by 2.0 M-NaCl. (a), (d) and (f) represent those polypeptides obtained by low-salt (0.25 M-NaCl) washes of native DNA-cellulose columns; (b), (e) and (g) represent those polypeptides solubilized by high-salt (2.0 M-NaCl) washes of native DNA-cellulose columns; (c) and (h) represent polypeptides eluted from denatured DNA-cellulose columns with low-salt (0.25 M-NaCl) or high-salt (2.0 M-NaCl) washes respectively.

leading to new gene transcription. Because of this evidence and the existence of data supporting a role for phosphorylation in transcription (for review see Jungmann & Kranias, 1977), interest has focused on the non-histone nuclear proteins.

Cyclic AMP has been a complicating factor in this puzzle. All cellular effects of cyclic AMP are thought to be mediated via cyclic AMP-dependent protein kinases (Kuo & Greengard, 1972); increases in cyclic AMP can lead to induction of various enzymes, possibly involving translocation of the cytoplasmic cyclic AMP-dependent protein kinase (Jungmann & Kranias, 1977), and cyclic AMP can act both to inhibit (for review see Abell & Monahan, 1973) and to stimulate (Green, 1978; Pawelek, 1979) cell proliferation. Thus phosphorylation of nuclear proteins has the potential of occurring via cyclic nucleotide-dependent and -independent mechanisms,

and either type may be involved in gene activation or cell proliferation, or both. Because of our previous work investigating the cell-growth-related expression of cyclic AMP-dependent and -independent protein kinases (Costa *et al.*, 1975, 1976, 1977, 1978), cell-cycle-related changes in nuclear protein synthesis (Gerner & Humphrey, 1973; Gerner *et al.*, 1976) and phosphorylation of nuclear and DNA-binding protein (Magun *et al.*, 1975; Magun, 1979), we considered it was now possible to ask two rather simple questions. They include: (1) are there cyclic AMP-independent growth-associated changes in the phosphorylation of nuclear proteins and can these be related to known changes in the expression of cyclic AMP-independent protein kinases? (2) are there equivalent changes in cyclic AMP-dependent phosphorylation mechanisms and what correlations exist?

The results given in the present paper demonstrate cell-growth-related differences in both cyclic AMP-dependent and -independent phosphorylation of nuclear proteins. In general, nuclear proteins are phosphorylated to a smaller degree in quiescent cultures than in exponential-phase cultures. The major component of nuclear proteins in CHO cells, namely the fraction insoluble in 0.3 M-NaCl, which contains predominantly DNA-binding proteins, only shows enhanced phosphorylation in response to cyclic AMP when cultures are in exponential-phase growth. There are specific differences in both cyclic AMP-dependent and -independent mechanisms. A major polypeptide of mol.wt. 31 000 in the 0.6 M-NaCl-soluble fraction, which appears to be a double-stranded-DNA-binding protein, is phosphorylated in a cyclic AMP-independent manner in exponential-phase cells. In quiescent cultures this peptide is not phosphorylated, in either a cyclic AMP-dependent or -independent manner. A polypeptide of mol.wt. 38 000 in the 0.3 M-NaCl-soluble fraction shows specific enhancement of phosphorylation in response to cyclic AMP in both exponential-phase and plateau-phase cells. This finding is consistent with previous work that has shown specific phosphorylation of non-histone proteins by cyclic AMP-dependent mechanisms (Hirsch & Martelo, 1976; Kraniias *et al.*, 1977).

Since we show that the polypeptides in exponential-phase and plateau-phase cultures are qualitatively similar, and since all nuclear protein fractions can act as substrates for cyclic AMP-dependent kinases *in vitro*, our data argue for regulation of phosphorylation of the 0.3 M-NaCl-insoluble proteins at the level of the protein kinase. This is consistent with our previous work showing that plateau-phase cultures are deficient in the type II cyclic AMP-dependent protein kinase (Costa *et al.*, 1978). Further, when these quiescent cells were stimulated to proliferate, this form of the enzyme was re-expressed within 5 h, before cellular entry into S-phase (Costa *et al.*, 1978). Others have also reported on phosphorylation control at the level of the kinases (Blat *et al.*, 1976). We would argue for control of the cyclic AMP-dependent phosphorylation of the 0.3 M-NaCl-insoluble nuclear proteins at the level of the type II form of protein kinase, which we have previously shown is under translational control as cells move through late G₁-phase (Costa *et al.*, 1976).

Since our results suggest that the polypeptides phosphorylated in response to cyclic AMP in the 0.3 M-NaCl-soluble fractions are not DNA-binding proteins and that the phosphorylation is independent of cell proliferation, we would argue that these proteins may be phosphorylated in the cytoplasm by the type I form of protein kinase and then transported to the nucleus. This would afford a

degree of regulation at the level of the substrate, in that the type I enzyme would not be translocated and the type II enzyme would only phosphorylate DNA-binding proteins, possibly utilizing the DNA in the enzymic process.

Our results on cyclic AMP-independent phosphorylation also argue for some regulation at the enzyme level. Here we show that quiescent cells, which have a G₁-phase-like DNA content, display lower degrees of phosphorylation than do exponential-phase cultures. We have previously shown that cells in early G₁-phase express minimum values of cyclic AMP-independent protein kinase activity and that this increases as cells move into and through S-phase (Costa *et al.*, 1977). We do not disregard regulation occurring at the substrate, or other, level. That this must occur is evident, in that not all nuclear protein fractions are phosphorylated to the same degree. The degree of phosphorylation does not, however, correlate with DNA-binding activity.

In summary, the data presented demonstrate that DNA-binding proteins are phosphorylated in a cell-proliferation-dependent fashion in response to cyclic AMP. Since this phosphorylation does not occur in quiescent yet genetically active cells, we would argue that this phosphorylation is related to some chromatin structural change involved in cell replication. In contrast, nuclear proteins that show little or no DNA-binding activity are actively phosphorylated, and this covalent modification is dependent on cyclic AMP but independent of cell replication. Since others have shown that nuclear proteins soluble in 0.35 M-NaCl may be responsible for the functional characteristics of chromatin (Kostraba *et al.*, 1975) and that specific enzymes can be induced in response to elevations in cyclic AMP concentration (e.g. see Byus & Russell, 1975), our data would be consistent with the possibility that phosphorylation of 0.3 M-NaCl-soluble nuclear proteins may be involved in gene activation. This mechanism would presumably involve some protein-protein interaction to facilitate transcription, since increased phosphorylation does not result in an increase in DNA-binding activity of these proteins.

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