Reversible Phosphorylation of a Nucleosome Binding Protein That Stimulates Transcription of Nucleosome Deoxyribonucleic Acid†

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ABSTRACT: A nonhistone chromosomal protein (D-55) of M_r 55 000 has been isolated in homogeneous form from calf thymus by using the standard salt-extraction procedures for the isolation of nonhistone chromosomal proteins followed by hydroxyapatite chromatography. D-55 is further characterized as coming from the group of nonhistone chromosomal proteins easily phosphorylated by an endogenous nuclear protein kinase. The kinase incorporates 1 mol of phosphate per mol of protein from [γ-^{32}P]ATP. The unphosphorylated form of D-55 binds to DNA, histones, and nucleosomes. Phosphorylation of D-55 does not significantly alter the binding of D-55 to DNA but greatly enhances its binding to histones and nucleosomes. Binding of D-55 to reconstituted nucleosomes enhances transcription of the nucleosome DNA by *E. coli* RNA polymerase by ~100-fold, to a level ~4 times that observed with naked calf thymus DNA as template. Phosphorylation of D-55 abolishes this enhancement. Binding of D-55 produces no apparent alteration in nucleosome structure as assayed by nuclease digestion patterns. In contrast, phospho-D-55 alters nucleosome structure significantly.

Phosphorylation of eukaryotic nuclear proteins has been shown to accompany such gross changes in cell structure and function as those occurring during growth and development, hormonal stimulation, and malignant transformations (Kleinsmith, 1975). Thus, it has been reasonable to assume that phosphorylation–dephosphorylation of various nuclear proteins may be one of the molecular mechanisms by which the cell controls gene expression (Gershey & Kleinsmith, 1969). Of the various classes of nuclear proteins which undergo phosphorylation, the group known as the acidic nonhistone chromosomal proteins has been observed to be particularly rich in phosphate groups (mainly phosphorylated seryl and threonyl residues) and to display the greatest variation in phosphorylation from tissue to tissue and in response to changes in conditions of cell growth and development (Teng et al., 1971). Variations in the quantity and the level of phosphorylation of the nonhistone chromosomal proteins during the cell cycle have been well documented (Borun & Stein, 1972; Ruiz-Carrillo et al., 1974; Yeoman et al., 1975). The nucleus contains a variety of protein kinases capable of phosphorylating nonhistone chromosomal proteins (Kranias & Jungmann, 1978). The nuclear sap also has phosphatases that will dephosphorylate this same group of proteins. Using the standard salt-extraction procedures for isolating the nonhistone chromosomal proteins from calf thymus nuclei, we have isolated from the fraction binding to hydroxyapatite a 55 000-dalton protein, D-55, which is phosphorylatable by an endogenous nuclear protein kinase and which binds to DNA and histones in both the phospho and dephospho forms. The protein is present at $\sim 10^5$ copies per cell and in the dephospho form binds to calf thymus nucleosomes and stimulates transcription of nucleosome DNA by *E. coli* RNA polymerases to ~100 times the rate observed for reconstituted nucleosomes in low salt. Phosphorylation of D-55, one phosphoryl group per monomer, abolishes this transcriptional stimulation and results in much tighter binding of the protein to the nucleosome as well as to isolated histones.

Materials and Methods

Protein Isolation. All procedures except as noted were carried out at 4 °C. Calf thymus glands (~1 kg) obtained immediately after slaughter were freed of connective tissue and major blood vessels. The tissue was minced with scissors and ground in a Waring blender in 3 volumes of buffer A (10 mM Mops,[1] 10 mM NaCl, 1 mM β-ME, 1 mM PMSF, and 1% ethanol, pH 7.4) in 1-min bursts with cooling in between until thoroughly dispersed. The material was filtered through several layers of cheesecloth prewashed with buffer A and centrifuged at 30 000*g*. The pellet was twice resuspended in buffer A and centrifuged. The final pelleted material was dissolved in an equal volume of buffer B (20 mM Mops, 3.99 M NaCl, 20 mM KH_2PO_4, 1 mM β-ME, 10% glycerol, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4). Approximately 10 volumes of buffer C (20 mM Mops, 2 M NaCl, 10 mM EDTA, 1 mM KH_2PO_4, 1 mM β-ME, 5% glycerol, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4) were added, and the solution was stirred overnight. Solid poly(ethylene glycol) 6000 was added to a final concentration of 10%, and the solution was stirred 2 h. The precipitated nucleic acids were removed by centrifugation at 30 000*g*.

The supernatant was filtered through Whatman No. 1 filter paper and chromatographed on a 4 × 20 cm hydroxyapatite (Bio-Rad HTP) column equilibrated with buffer C; the column was washed with buffer until the eluant had $A_{278} = 0$. The column was eluted in two steps, 50 mM phosphate and 300 mM phosphate in buffer C, allowing the A_{278} of the wash to return to the base line in between steps. The flow through is termed fraction A, the proteins eluted with 50 mM phosphate are denoted as fraction B, and the protein eluted with 300 mM phosphate is termed fraction C.

The fraction B or C proteins were prepared for DNA–cellulose chromatography by successive dialyses against 20 mM Mops, 10 mM EDTA, 1 mM β-ME, 10% glycerol, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4, that contained 1, 0.5, 0.25, or 0.05 M NaCl. This will be referred to as buffer D with the NaCl concentration indicated. The protein was then

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[1] Abbreviations used: β-ME, β-mercaptoethanol; Mops, 4-morpholineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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loaded onto sequential columns: cellulose (10 mL), followed by four 50-mL columns by DNA–cellulose made of double-stranded salmon sperm DNA, double-stranded calf thymus DNA, single-stranded salmon sperm DNA, and single-stranded calf thymus DNA, respectively. The DNA–cellulose columns were prepared by the method of Alberts & Herrick (1971), with two cycles of drying to increase the amount of DNA immobilized on the cellulose. After the samples were loaded, the columns were separated, and the DNA binding proteins were eluted from each column separately with buffer D containing 2 M NaCl. The first column served as a control to eliminate proteins that adsorb nonspecifically to cellulose. The salt was removed from the eluates by step-gradient dialysis as above and finally dialyzed vs. buffer E (20 mM Mops, 10 mM EDTA, 1 mM β-ME, 50 mM NaCl, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4).

After elution of the fraction B proteins from salmon sperm double-stranded DNA, the protein identified below as D-55 was purified further by gel filtration by the use of a 5 × 100 cm column of Bio-Gel P200 (Bio-Rad) with a flow rate of 10 mL/h. The protein designated S-70 was similarly purified from the fraction B proteins which bound to the calf thymus single-stranded DNA. Proteins were stored in buffer F (20 mM Mops, 1 mM EDTA, 1 mM β-ME, 10% glycerol, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4) at −20 °C and appeared to be stable for 2–3 months. No protease activity was observed as determined by electrophoretic analysis of the stored proteins.

Kinase Reaction. The hydroxyapatite column fraction A, B, or C proteins or the eluate of fraction B proteins from salmon sperm double-stranded DNA–cellulose contained an endogenous kinase activity. The fractions were exhaustively dialyzed vs. buffer G (20 mM Mops, 1 mM β-ME, 0.1 mM PMSF, and 0.1% ethanol, pH 7.4). Phosphorylation was achieved by the addition of $MgCl_2$ to 20 mM and ATP to 10^{-5} M followed by incubation at 37 °C for 15 min. The reaction was stopped by addition of excess EDTA and rapid chilling. Phospho-D-55 could be purified from the phosphorylated fraction B proteins by DNA–cellulose chromatography and gel filtration as described above or from the phosphorylated salmon sperm double-stranded DNA–cellulose binding proteins of fraction B by gel filtration.

Test reactions were with 0.1 mL of protein in buffer G containing 1 μCi of [γ-^{32}P]ATP (3 Ci/mmol) and various concentrations of $MgCl_2$ or $MnCl_2$. Aliquots (0.05 mL) of the reaction mixtures were spotted on Whatman 3 MM filter disks to determine the stoichiometry of phosphorylation. The disks were washed with cold 5% Cl_3AcOH, followed by three 15-min washes with 2% Cl_3AcOH–0.01 M $Na_4P_2O_7$. The filters were washed twice with ethanol–ether (1:1) and once with ether, dried, and then counted in 10 mL of scintillation fluid (Amersham ACS) in a Packard Model 3002 liquid scintillation spectrometer.

Dephosphorylation Reaction. Solutions of *E. coli* alkaline phosphatase or alkaline phosphatase attached to Sepharose beads were kindly supplied by Dr. J. F. Chlebowski. Dephosphorylation of proteins was in buffer G containing 1 mM $ZnCl_2$. Enzyme, 5 μg, was added to 0.1 mL of protein and incubated at 37 °C for 30 min.

Gel Electrophoresis. All chemicals used were Bio-Rad electrophoresis purity reagents. Polyacrylamide gels were similar to those described by Laemmli (1970). Gels were stained by soaking in 0.25% Coomassie Blue R-250 in 45% ethanol–9% acetic acid for 1 h. Gels were destained by soaking in 40% ethanol–10% acetic acid for several hours or by electrophoresis at 25 V across the thin dimension of the gel with the same destaining solution.

Isoelectric focusing was in 7.5% acrylamide slab gels containing 1:39 bisacrylamide cross-linker, 1% Biolyte 4/6, 1% Biolyte 5/7, and 0.5% Biolyte 3/10 (Bio-Rad). Buffer reservoirs were 0.06 N H_2SO_4 and 0.04 N NaOH–0.02 N $Ca(OH)_2$. After preelectrophoresis for 30 min at 200 V, the gels were loaded with the samples at the acidic end and then run for 20 h at the same voltage. The pH gradient was determined by cutting out 2-mm pieces from the center track and soaking in 0.5 mL of H_2O. These gels were stained and destained as above.

The TBE gels used for electrophoresis of DNA were based on the procedure of Maniatis et al. (1975). The DNA bands were visualized by autoradiography or by staining with ethidium bromide (1 μg/mL in buffer H). Coomassie Blue stained gels, photograph negatives of stained gels, or autoradiographs were scanned with a Joyce–Loebl densitometer. The high sensitivity of the latter instrument makes it more effective in visualizing minor bands than photographic reproduction.

Amino Acid Analysis. Protein, 0.25 nmol, was acid hydrolyzed for 20 h at 110 °C in 6 N HCl saturated with phenol. The hydrolysate was analyzed on a Beckman Model 121-M amino acid analyzer. Cysteine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan content was determined by the spectroscopic method of Edelhoch (1967).

Circular Dichroism. Circular dichroism spectra were measured in a Cary 61 spectropolarimeter by using protein concentrations of about 0.1 mg/mL and a path length of 0.3 cm. Results were expressed as the mean residue ellipticity with a mean residue weight of 110.

Melting Temperature Profiles. The hyperchromicity at 260 nm of the poly[d(A-T)] in buffer F containing D-55 or S-70 was measured in a Cary 118 recording spectrophotometer with a full scale of 0.2 OD units. Temperature was varied at a rate of 1 °C/3 min by using a circulating water bath and jacketed cuvettes. Both helix → coil transitions with increasing temperature and coil → helix transitions with decreasing temperature were monitored.

Labeling of DNA. Whole T7 DNA and the T7 DNA restriction fragment known as *Hpa*II Cl were the generous gift of Dr. R. Strothkamp. Whole T7 DNA was labeled with ^{32}P by phosphorylating the 5′ ends as previously described (Maxam & Gilbert, 1977). Sonicated calf thymus DNA was labeled by incorporating ^{32}P or ^{3}H nucleotides into single-stranded regions. A total of 50 μg of DNA was incubated for 1 h at 25 °C in 0.1 mL of 50 mM Tris, 5 mM $MgCl_2$, 1 mM β-ME, and 0.05 mg/mL BSA, pH 7.8, containing 6 units of *E. coli* DNA polymerase (Boehringer–Mannheim), 5 μM each dGTP, dCTP, and TTP, and 120 μCi of [α-^{32}P]dATP (350 Ci/mmol) or [8-^{3}H]dATP (35 Ci/mmol). After extraction with phenol, the DNA was electrophoresed and recovered as above. Poly[d(A-T)] and T7 Hpa II Cl were labeled by base substitution. The reaction was the same as for the calf thymus DNA except that 10^{-10} g of pancreatic DNAse (Sigma) was included in the reaction which was carried out at 15 °C. After being labeled, the DNAs were diluted with unlabeled DNA to give specific activities on the order of 10^4 cpm/μg. Single-stranded DNA was made by heat denaturation of native DNA at 100 °C for 15 min in 10 mM Tris and 1 mM EDTA, pH 7.5, followed by rapid freezing.

Filter Binding Assays. Purified D-55, phospho-D-55, and S-70 were exhaustively dialyzed vs. buffer F. Protein and labeled DNA were incubated in 0.1 mL of buffer F containing

50 μg of BSA and varying amounts of NaCl, $MgCl_2$, or heparin. After incubation for 20 min at 37 °C, the reaction mixture was spotted on a nitrocellulose filter (Millipore HWAP02500), and the filter was then washed with 1 mL of buffer containing the same concentrations of NaCl, $MgCl_2$, or heparin. The filters were counted in 10 mL of scintillation fluid (Amersham ACS). All reactions were done in triplicate. The data shown have been corrected for differences in the efficiency of retention of protein–DNA complexes on the filter in order to standardize the assays. The efficiency, dependent on the DNA size, was determined by measuring the amount of DNA retained in the presence of excess protein.

Histone–Sepharose Chromatography. Preparation of histone–Sepharose was based on the method of March et al. (1974). Sepharose 6B (Pharmacia), 20 mL of suspension, was washed repeatedly with H_2O and then suspended in 20 mL of 2 M Na_2CO_3. Cyanogen bromide, 2 mL in acetonitrile (1:1), was added and vigorously stirred for 2 min on ice in a hood. The slurry was decanted onto a coarse sintered-glass funnel and washed repeatedly with 0.1 M $NaHCO_3$ (pH 9.5), then with H_2O, and finally with 0.1 M $NaHCO_3$ (pH 8.5). After the final wash, the Sepharose slurry was dried to a moist cake and mixed with 20 mL of histone solution (0.5 mg/mL in 0.1 M $NaHCO_3$, pH 8.5). The mixture was gently agitated at 4 °C for 20 h, 100 mL of 0.1 M Tris-HCl, pH 7.4, added, and the mixture allowed to sit at room temperature for 30 min. The histone–Sepharose was then washed with several aliquots of the 0.1 M Tris. Over 95% of the added histone remains bound to the Sepharose under these conditions.

A column, 3-mL packed volume, containing approximately 3 mg of histone was equilibrated with buffer F and used for chromatography. A control column of Sepharose prepared in the same manner but incubated in buffer without histone was employed to test for non-histone-specific binding. After the columns were loaded, they were eluted with increasing salt steps in buffer F. The eluted protein was analyzed electrophoretically to rule out release of histones.

Polymerase Assays. E. coli RNA polymerase was the generous gift of Dr. E. Spicer. One unit of enzyme at 37 °C, pH 7.9, in 0.1 mL of standard assay solution (Burgess, 1969) and containing 1 μCi of [^{3}H]UTP (41 Ci/mmol) and the DNA templates ±D-55 and phospho-D-55 as described in Results was incubated for 10 min. The reactions were stopped by rapid chilling, and 75 μL of each was spotted on a Whatman 3 MM filter disk and treated as described above for the protein kinase reaction.

Nucleosome Binding. Nucleosomes were reconstituted according to the procedure of Camerini-Otero et al. (1976). Total calf thymus histone and sonicated DNA, 1 mg each, were mixed in 2 mL of 5 M urea, 2 M NaCl, and 1 mM Tris, pH 8.0. The solution was dialyzed successively against 1.2, 1.0, 0.8, and 0.6 M NaCl in 5 M urea, 10 mM β-ME, and 1 mM Tris, pH 8.0. The urea was removed by dialysis against 0.6 M NaCl and 1 mM Tris, pH 8.0, and then against 1 mM Tris, 0.1 mM Tris, and 0.1 mM EDTA, pH 8.0. Fidelity of reconstitution was checked by staphylococcal nuclease or pancreatic DNAse digestion by the methods of Camerini-Otero et al. (1976).

For measurements of [^{32}P]phospho-D-55 binding to nucleosomes, reconstituted nucleosomes, [^{32}P]phospho-D-55, and varying concentrations of D-55 were incubated in 0.67 mL of buffer F at room temperature for 15 min. The nucleohistone was precipitated by addition of 30% polyethylene glycol in buffer F to a final concentration of 10%. After being vortexed, the mixture was centrifuged at 15000*g* for 10 min. The supernatant was withdrawn, and the pellet was resuspended in 10 mL of scintillation fluid and counted.

Results

Protein Isolation. Fraction A, the flowthrough from hydroxyapatite chromatography, consisted mainly of histones and a few other species, probably basic in nature. Fraction B, eluted with 50 mM phosphate, contained the majority of the acidic proteins and trace amounts of both histone H4 and one of the two major forms of histone H1. Fraction C contained several of the same species as fraction B, possibly in more highly phosphorylated form. The gels of these fractions are shown below after separation on DNA–cellulose columns. Since we were interested in finding a phosphorylatable chromatin-binding protein, the first criterion used for isolation was binding to DNA–cellulose.

The DNA binding proteins of fraction B were isolated by chromatography on sequential DNA–cellulose columns of double-stranded heterologous and homologous DNA and single-stranded heterologous and homologous DNA. This permitted separation of the proteins specifically binding to single-stranded DNA. The purpose of using homologous DNA–cellulose columns as well as heterologous DNA–cellulose columns was twofold. First, although it was expected that proteins with a high degree of DNA sequence specificity would be present in too small a quantity to detect, we wanted to allow for that possibility. Second, the sizes of the heterologous DNA–cellulose columns were chosen so that the protein binding capacity of each would be approximately half that needed for retention of all the DNA binding proteins. This allowed separation of proteins with higher binding affinities from those with lower affinities for the same form of DNA. The preferential binding of the proteins in fractions B and C to the various DNAs is illustrated by the $NaDodSO_4$ gels, stained with Coomassie Blue, after elution from the DNA–cellulose columns (Figure 1).

Calf thymus single-stranded DNA binds the largest number of protein species from both fractions B and C. Homologous and heterologous double-stranded DNA binds far fewer species. The most prominent in the latter group is a protein of M_r 55000, designated D-55, which binds to both double- and single-stranded DNA (Figure 1). A second protein contained in fraction B appears to bind exclusively to single-stranded DNA under the conditions of the DNA–cellulose chromatography and has a molecular weight of ~70000, designated S-70 (Figure 1).

Proteins D-55 and S-70 were both isolated in homogeneous form by further chromatography of the eluates from the DNA–cellulose columns on Bio-Gel P200 columns. Densitometer traces of the gels of the homogeneous proteins stained with Coomassie blue will be shown below. From 1 kg of thymus, estimated to contain 4.6×10^{12} cells (Bollum, 1975), we isolated approximately 100 mg of each of the proteins described here. From this, it was estimated that there are at least 10^5 copies of each protein per cell.

Phosphorylation of the Nonhistone Proteins in Fraction B by an Endogenous Nuclear Protein Kinase. The fractions from the hydroxyapatite column contain a copurifying protein kinase activity, since incubation of the pooled fractions in buffer G containing $MgCl_2$ and [γ-^{32}P]ATP results in the incorporation of radioactive label into the majority of the proteins in each fraction. This endogeneous nuclear protein kinase activity associated with fraction B was optimal at a Mg^{2+} concentration of 20 mM. Mn^{2+} gave a maximal activity at 15 mM, but this activity was approximately half that shown by Mg^{2+}. In the standard reaction mixture with 20 mM $MgCl_2$, incorporation

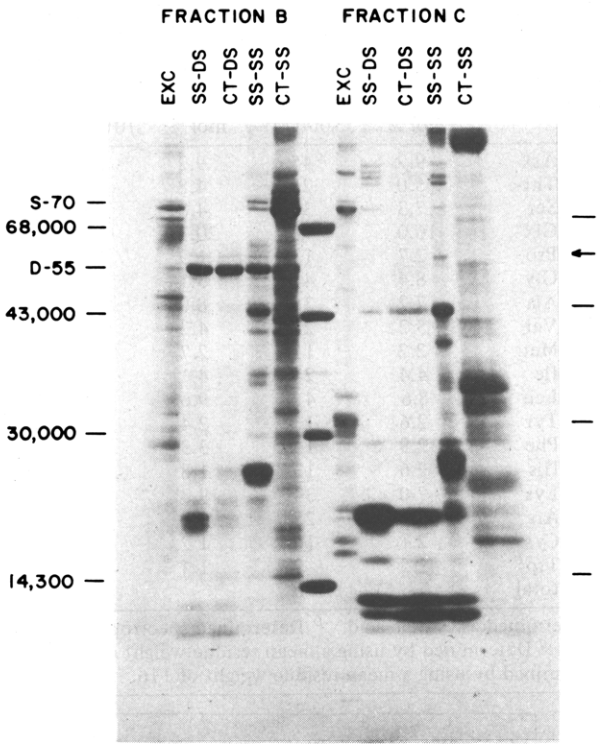

FIGURE 1: Acrylamide gradient (5–20%) $NaDodSO_4$ gel of the DNA–cellulose binding proteins of fractions B and C stained with Coomassie Blue. An aliquot, ~30 μg, of each DNA–cellulose binding fraction was analyzed. The protein designated D-55 occurs prominently in the salmon sperm double-stranded DNA binding fraction but is also present in the others, while S-70 is limited to the calf thymus single-stranded DNA binding fraction. The arrow shows the position of a protein of molecular weight ~55 000 in fraction C. EXC, excluded fraction; SS-DS, salmon sperm double-stranded DNA binding proteins; CT-DS, calf thymus double-stranded DNA binding proteins; SS-SS, salmon sperm single-stranded DNA binding proteins; CT-SS, calf thymus single-stranded DNA binding proteins.

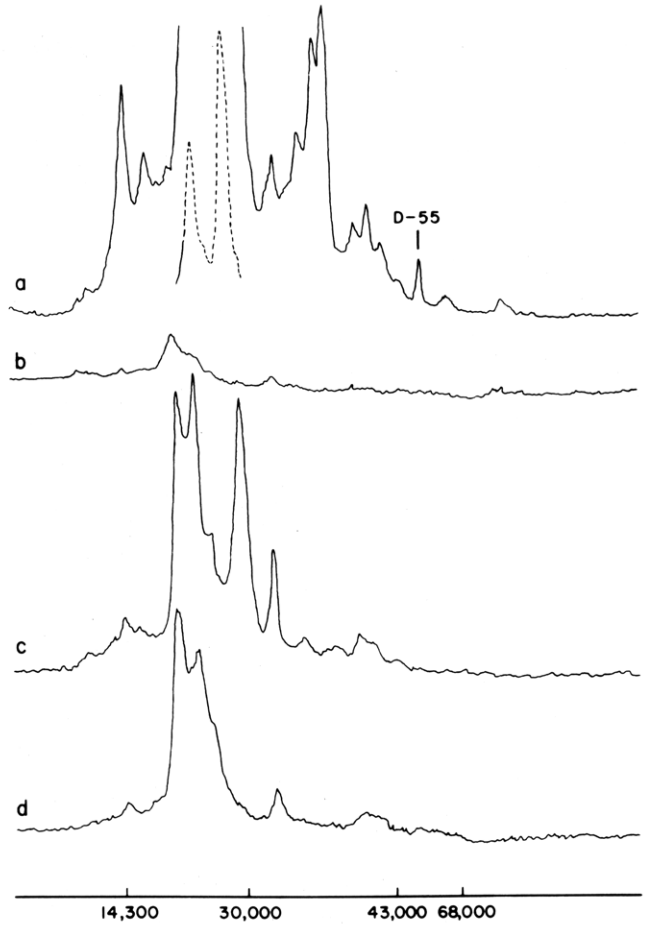

FIGURE 2: DNA–cellulose chromatography of phosphorylated fraction B proteins. The fraction B proteins were dialyzed into buffer D after phosphorylation and chromatographed on the sequential DNA–cellulose columns as described. The DNA binding proteins were analyzed on a 5–20% acrylamide-gradient $NaDodSO_4$ gel and the labeled proteins detected by autoradiography. Shown are densitometer tracings of the tracks on the autoradiograph corresponding to (a) salmon sperm double-stranded DNA–cellulose, (b) calf thymus double-stranded DNA–cellulose, (c) salmon sperm single-stranded DNA–cellulose, and (d) calf thymus single-stranded DNA–cellulose. No detectable proteins bound to the control of plain cellulose. In contrast to Figure 1, the capacity of column a was sufficient to bind all of the phospho-D-55.

of label reached a maximum after 10–15 min and decreased slowly over a 2-h period. The patterns of protein phosphorylation for the fraction B proteins binding to the various DNA–cellulose columns are shown in Figure 2 by the densitometer traces of the radioautographs of the $NaDodSO_4$ gels of each fraction following incubation with [γ-^{32}P]ATP.

Substrates of the Kinase. Protein D-55 was phosphorylated by the copurifying kinase activity in fraction B as demonstrated by electrophoretic analysis of the ^{32}P-labeled proteins after binding and elution from a salmon sperm double-stranded DNA–cellulose column (Figures 2a and 3a). Double-stranded calf thymus DNA could have been used as well. Protein S-70, however, could not be phosphorylated under the conditions used, as shown by the complete absence of a band corresponding to S-70 in the radioautographs of the gels from both single-stranded DNA–cellulose columns (Figure 2c,d). Prior treatment of the fraction B proteins with alkaline phosphatase–Sepharose did not enable protein S-70 to be phosphorylated. The total amount of label incorporated in the other proteins, however, was increased by up to three times.

Dephosphophosvitin was a poor substrate for the kinase activity. Addition of histones to the kinase reaction resulted in an increased incorporation of label directly proportional to the amount of histone added. Electrophoresis of the labeled proteins on a 10% acrylamide–$NaDodSO_4$ gel clearly demonstrated that this increase was due to the phosphorylation of the added histones. DNA added to the kinase reaction was found to inhibit protein phosphorylation. In all reactions, 1 μM cAMP had no effect either on the extent of phosphorylation or on the ability of a particular protein species to be labeled.

Isolation of Phospho-D-55. After phosphorylation, the fraction B proteins can be chromatographed on DNA–cellulose columns in the usual manner as illustrated above. No detectable protein bound to the control of plain cellulose. Since a labeled protein corresponding to D-55 was found associated with the salmon sperm double-stranded DNA–cellulose (Figure 3a), the DNA binding behavior of the phosphorylated protein must be similar to that of the unmodified D-55.

The salmon sperm double-stranded DNA–cellulose binding proteins from unmodified fraction B retain protein kinase activity after elution from the DNA–cellulose column. Incubation of these proteins under the phosphorylation conditions used for total fraction B also resulted in phosphorylation of protein D-55 as shown by the radioautograph in Figure 3a which is of the gel made after rechromatography of the eluate from the double-stranded DNA column on a second double-stranded DNA–cellulose column. The ratio of phosphoryl groups per molecule of D-55 was ~1 as calculated from the specific activity of the label and the protein concentration of the isolated phospho-D-55. The experiments to be described

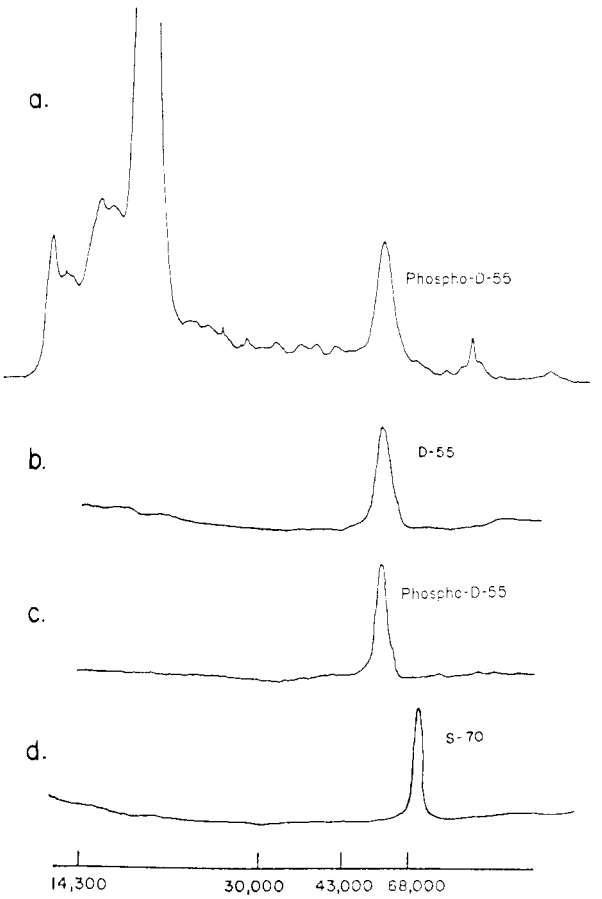

FIGURE 3: (a) Densitometer tracing of an autoradiograph of a 10% acrylamide $NaDodSO_4$ gel of the phosphorylated salmon sperm double-stranded DNA–cellulose binding proteins. Proteins were phosphorylated in buffer G containing 20 mM Mg^{2+} and [γ-^{32}P]ATP. Densitometer tracings of the 12% acrylamide–$NaDodSO_4$ gels stained with Coomassie Blue of purified proteins D-55 (b), phospho-D-55 (c), and S-70 (d).

below used phospho-D-55 purified by further gel filtration of these phosphorylated salmon sperm double-stranded DNA binding proteins. A denistometer trace of the $NaDodSO_4$ gel of the purified phospho-D-55 stained with Coomassie Blue is shown in Figure 3c. Densitometer traces of the gels of the corresponding homogeneous preparations of unphosphorylated D-55 and S-70 stained with Coomassie Blue are shown for comparison in Figure 3b,d.

Characterization of Nonhistone Chromosomal Proteins D-55 and S-70. The amino acid compositions of proteins D-55 and S-70 are shown in Table I. Characteristic of the acidic nonhistone chromosomal proteins, both have a large number of Asx and Glx residues. By isoelectric focusing, D-55 has an isoelectric point of 4.5 ± 0.2 while S-70 has a p*I* of 5.1 ± 0.2, suggesting that many of the Asx and Gsx residues are present in the carboxyl form. Ultraviolet circular dichroism in the region 250–200 nm indicates that both proteins contain relatively small amounts of α helix, ~8% for S-70 and 5% or less for D-55 as estimated by the method of Greenfield & Fasman (1969).

DNA Binding. Since proteins D-55 and S-70 were isolated by DNA–cellulose chromatography, they have at least some affinity for DNA. Further features of DNA complex formation were examined by the nitrocellulose filter binding technique. Unmodified D-55 causes the retention of both double-stranded and single-stranded DNAs on nitrocellulose filters, but at low salt concentrations D-55 is much more efficient in retaining double-stranded DNA (Figure 4a), as

Table I: Amino Acid Analysis of Proteins D-55 and S-70

	D-55		S-70	
	mol %	~residues/ 55 000 M^c	mol %	~residues/ 70 000 M^d
Asx	9.2	45	9.2	55
Thr	5.0	25	4.8	29
Ser	7.3	36	4.8	29
Glx	16.0	78	20.2	122
Pro	2.7	13	1.9	11
Gly	8.4	41	5.1	31
Ala	7.2	35	8.0	48
Val	5.2	25	4.2	25
Met	2.2	11	2.7	16
Ile	4.4	22	4.1	25
Leu	8.6	42	9.6	58
Tyr	2.6	13	2.4	14
Phe	2.9	14	3.3	20
His	2.6	13	1.8	11
Lys	7.0	34	9.3	56
Arg	5.1	25	6.0	36
Cys[a]	2.1	10	1.2	7
Trp[b]	1.5	7	1.4	8
total		490		602

[a] Determined as cysteic acid. [b] Determined spectrophotometrically. [c] Determined by using a mean residue weight of 112. [d] Determined by using a mean residue weight of 116.

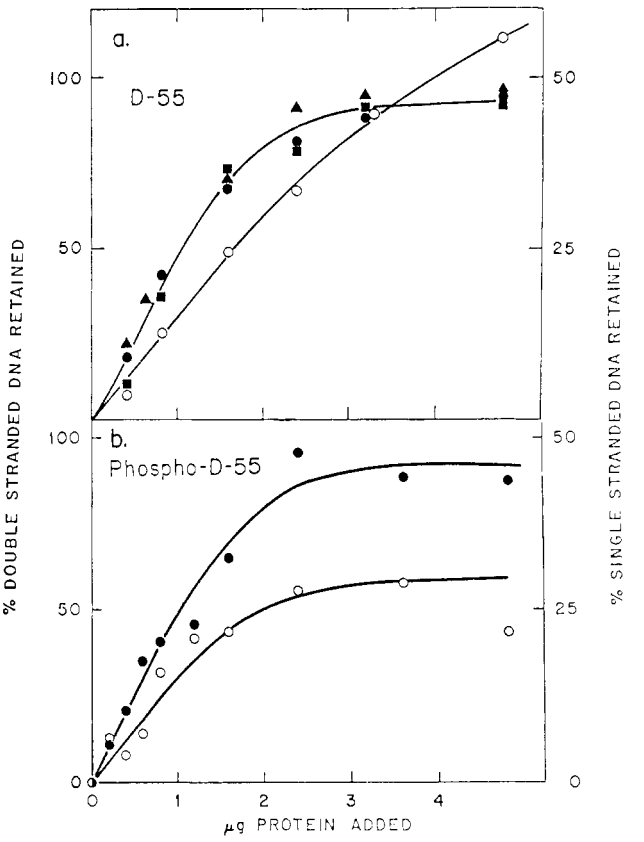

FIGURE 4: (a) Retention of DNA on nitrocellulose filters by protein D-55. ^{32}P-labeled double-standed DNA, 0.7 μg, was incubated in 0.1 mL of buffer F containing 50 mM NaCl and 50 μg of BSA with varying amounts of D-55. Binding to 0.7 μg of ^{32}P-labeled single-stranded DNA was in buffer containing no added salt. The results shown are the average of several experiments; standard deviations are on the order of 5%. Values have been corrected for the difference in binding efficiencies of different size DNAs. (●) Calf thymus double-stranded DNA; (■) whole T7 double-standard DNA; (▲) T7 Hpa II Cl double-stranded DNA; (○) calf thymus single-stranded DNA. (b) Retention on nitrocellulose filters of DNA by phospho-D-55. Increasing amounts of phospho-D-55 are incubated with ^{3}H-labeled double- or single-stranded calf thymus DNA and assayed as described. All standard deviations are less than 6%. (●) Double-stranded DNA; (○) single-stranded DNA.

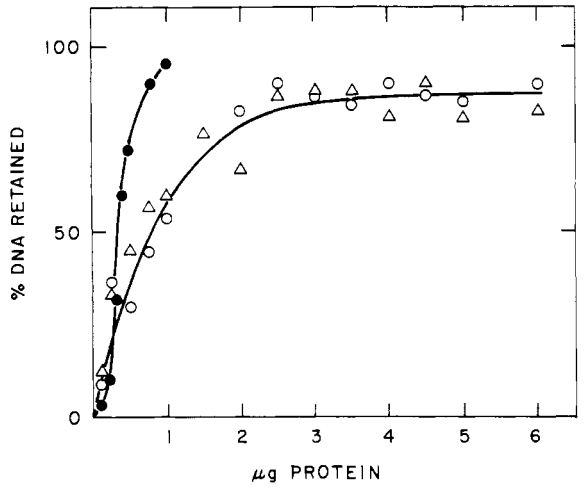

FIGURE 5: Retention of DNA on nitrocellulose filters by protein S-70. ^{32}P-labeled single-stranded DNA, 0.35 μg, or ^{32}P-labeled double-stranded DNA, 0.7 μg, was incubated in 0.1 mL of buffer F containing 50 μg of BSA and varying amounts of S-70. The results are the average of several experiments; standard deviations are less than 7%. Values have been corrected for differences in binding efficiencies. (●) Single-stranded calf thymus DNA; (△) single-stranded T7 *Hpa*II Cl; (○) double-stranded whole T7 DNA.

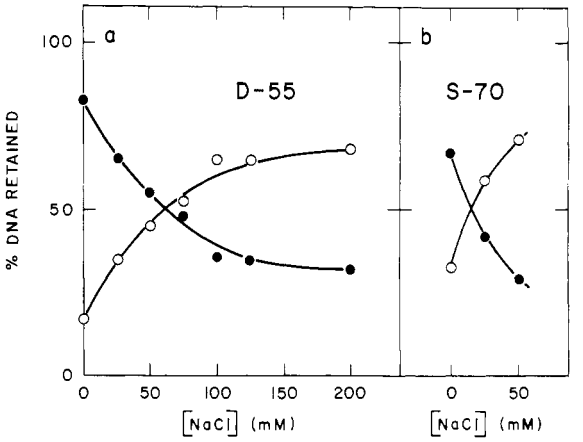

FIGURE 6: Specificity of the binding of D-55 and S-70 to double- or single-stranded DNA as a function of [NaCl]. ^{3}H-labeled double-stranded, 5 μg, and ^{32}P-labeled single-stranded, 2.5 μg, calf thymus DNA was incubated with (a) 2.4 μg of D-55 or (b) 3 μg of S-70 in 0.1 mL of buffer F containing 50 μg of BSA and increasing amounts of NaCl. Total binding of S-70 to DNA above 50 mM NaCl is too low to permit significant analysis. (●) Double-stranded DNA; (○) single-stranded DNA.

expected from its behavior on DNA–cellulose column chromatography (Figure 1). While binding to double-stranded DNA is predominant, filter binding shows that D-55 does have some affinity for single-stranded DNA (Figure 4a). Retention of double-stranded DNA on filters induced by D-55 shows the same protein concentration dependence for homologous calf thymus DNA or the heterologous T7 DNA (Figure 4a). This suggests that the binding site on the protein is not sequence specific. Retention of all the DNA on the filter also requires a protein to DNA weight ratio of ~3.4:1. This ratio applies no matter what the length of the DNA, since the same number of protein molecules is required for the retention of the same amount of DNA despite a length variation from 138 base pairs for the *Hpa*II Cl fragment of T7 to ~40 000 base pairs for whole T7 DNA (Figure 4a). The protein/DNA ratio of 3.4:1 required for saturation means that each D-55 must cover ~27 base pairs and that retention of all DNA on the filter requires almost full saturation of the binding sites present on each piece of DNA present. A possible explanation of this behavior is that cooperativity of binding exists and, therefore, each piece of DNA tends to become saturated before significant numbers of new sites nucleate on other DNA strands. As shown below, both D-55 and S-70 are proteins which bind comparatively weakly to DNA, and, therefore, the binding data cannot be interpreted in detail. Phosphorylation of D-55 has no effect on its binding to double-stranded DNA as assayed by the filter binding technique (Figure 4b). In contrast, the efficiency of its retention of single-stranded DNA is decreased by phosphorylation (Figure 4b).

At low ionic strength, S-70 retains both single-stranded and double-stranded DNA on the filters (Figure 5). Despite the fact that under the conditions of the DNA–cellulose column S-70 appears to prefer the single-stranded DNA (Figure 1), the filter binding assay shows it to have a slight preference for double-stranded DNA at very low ionic strength (see below).

The binding of both D-55 and S-70 to double-stranded DNA is rapidly inhibited by salt (Figure 6). Binding efficiency is inhibited approximately 50% by less than 0.1 M NaCl in both cases, suggesting that binding affinity is substantially less than sequence-specific proteins like RNA polymerase or *lac* repressor. Note that the protein/DNA ratios were adjusted in Figure 6 to give slightly less than 100% retention of the double-stranded DNA so that changes in affinity at the lowest salt concentrations could be detected. The salt dependence of binding reveals that while both proteins bind preferentially to double-stranded DNA at low salt concentrations, increasing salt actually enhances the affinities of both proteins for single-stranded DNA such that the preferences can be reversed at high enough NaCl concentrations (Figure 6). This effect is more pronounced for S-70 where binding affinities are equal for the two forms of DNA at ~15 mM NaCl, while equal binding affinities occur for D-55 at 75 mM. At no ionic strength, however, is there greater than at 10-fold preference for single-stranded over double-stranded DNA. The steep ionic strength dependence of this preference for S-70 would explain the apparent single-stranded DNA binding preference of S-70 as observed on the DNA–cellulose columns. Reflecting the generally weak binding to DNA is the finding that even under the conditions where binding to single-stranded DNA is preferred, neither D-55 nor S-70 alters the melting temperature profile of calf thymus DNA. This is in agreement with the estimate of Herrick & Alberts (1976) that proteins with only a 10-fold difference in affinity for single-stranded vs. double-stranded DNA would depress the melting temperature by less than 1 °C.

Histone Binding. While both of the proteins under study here show relatively low affinities for DNA, nonhistone chromosomal proteins may also bind to the highly positively charged histones present in the nucleosome. Binding affinity of D-55 and S-70 for histones was tested by passage of the homogeneous proteins over histone–Sepharose columns. At low salt concentration (0.1 M) S-70 passes right through such columns, while D-55 is retained. D-55 elutes from the histone–Sepharose column at 0.2 M NaCl (Figure 7a). In contrast to the lack of effect of phosphorylation on DNA binding, phosphorylation of D-55 substantially increases the affinity of D-55 for histones (Figure 7b). Elution of phospho-D-55 from the histone–Sepharose column requires ~0.7 M NaCl. A small amount of unmodified D-55 present in the phosphorylated sample elutes in the original 0.1 M fraction (Figure 7b). Binding of fraction B proteins to histone–Sepharose columns is not a general characteristic of this group of proteins, since ~70% of the total protein in fraction B passes

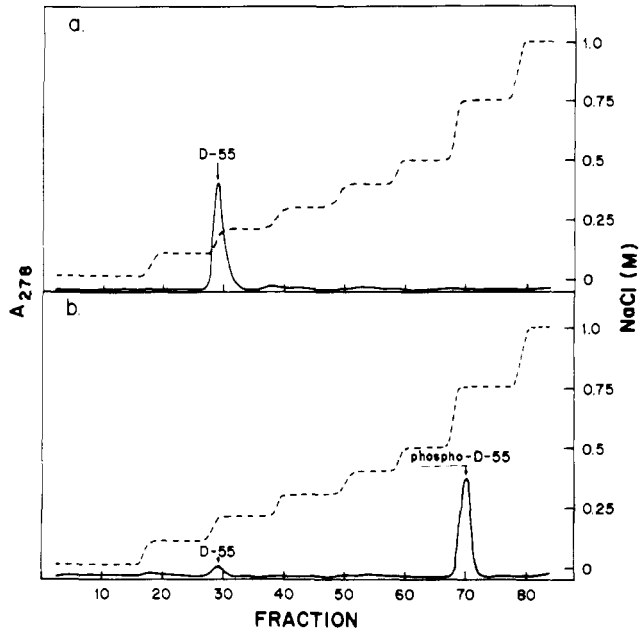

FIGURE 7: Histone–Sepharose chromatography of proteins D-55 and phospho-D-55. The proteins were chromatographed on a 3-mL column containing approximately 3 mg of total calf thymus histone. The column was eluted stepwise with buffer F containing increasing NaCl. The salt concentration of each fraction was monitored by its conductivity. (a) D-55; (b) phospho-D-55; (—) A_{278}; (– – –) NaCl concentration.

through the column at 0.1 M NaCl.

Nucleosome Binding of D-55. Since D-55 and its phosphorylated derivative bind to both components of the nucleosome, we determined if the protein has significant affinity for intact nucleosomes. Nucleosomes were prepared in reconstituted form from calf thymus DNA and calf thymus histones by the method of Camerini-Otero et al. (1976). Fidelity of reconstitution was ascertained by digestion of the nucleosomes with both pancreatic and staphylococcal nucleases followed by gel electrophoresis of the DNA. A densitometer trace of the ethidium bromide stained gel of the DNA fragments from a digest of the reconstituted nucleosomes by 10 μg of staphylococcal nuclease for 30 min is shown in Figure 8a and indicates the expected predominance of monomer (140 base pairs) and dimer (299 base pairs) DNA. At shorter intervals under the conditions used to monitor the effect of D-55 and phospho-D-55 on the digestion, staphylococcal nuclease produces a predominant monomer length of ~170 base pairs, as shown in Figure 8b.

Since phospho-D-55 can be radiolabeled with [^{32}P]ATP, the most convenient assay of complex formation with the nucleosome was to add the ^{32}P-labeled protein to the solution of nucleosomes and then use 30% poly(ethylene glycol) in buffer F to precipitate the nucleosomes. This treatment does not precipitate either D-55 or its phosphorylated form when free in solution. The precipitated pellet of nucleosomes was then resuspended in scintillation fluid and counted. A significant fraction of the added phosphorylated D-55 was found in the precipitated nucleosomes.

An assay of the binding of the unphosphorylated form was devised by using it to compete for nucleosome binding with the ^{32}P-labeled form. The nucleosomes containing bound [^{32}P]phospho-D-55 were incubated with gradually increasing concentration of D-55, and the amount of [^{32}P]phospho-D-55 remaining with the nucleosome was determined by precipitation (Figure 9). Approximately 25% of the nucleosome-bound phospho-D-55 is readily displaced by the unphosphorylated protein, while a second fraction is very poorly displaced. Only an additional 10% of phospho-D-55 is displaced from the nucleosome by 10 molecules of D-55 for each molecule of phospho-D-55 present (Figure 9). These two classes of bound phospho-D-55 may represent that bound rather loosely to the DNA spacers and that bound much more tightly to the core complex via the enhanced interaction of phospho-D-55 with the histone core.

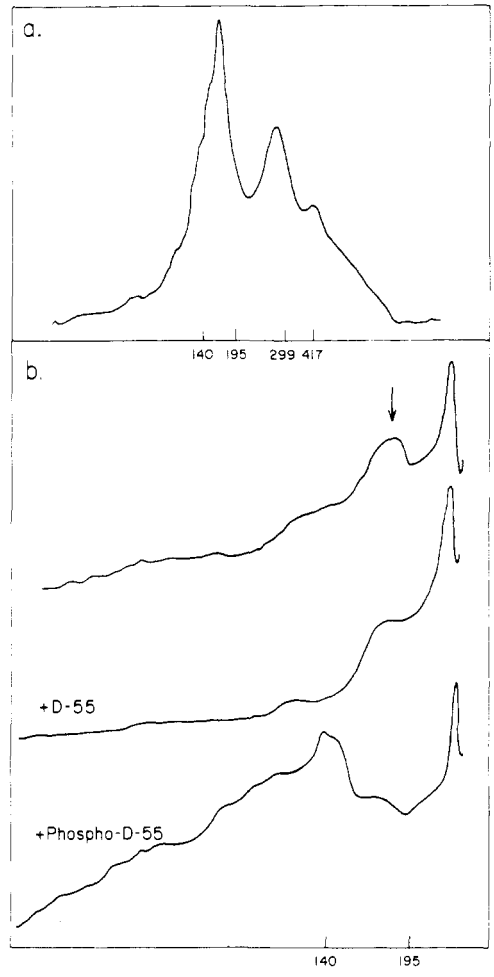

FIGURE 8: Densitometer tracings of acrylamide–TBE electrophoresis gels of the DNA fragments produced by nuclease digestion of reconstituted nucleosomes stained with ethidium bromide. (a) The characteristic multimeric lengths of DNA produced by staphylococcal nuclease digestion of nucleosomes; 10 μg of enzyme for 30 min at 37 °C, 6% acrylamide TBE gel. (b) Staphylococcal nuclease digestion patterns from reconstituted nucleosomes containing D-55 and phospho-D-55. The arrow shows the position of the normal-sized monomer DNA produced by the digestion conditions used: 1 μg of enzyme for 15 min at 37 °C, 10% acrylamide–TBE gel.

Stimulation of Transcription of Nucleosome DNA by D-55. The double-stranded DNA incorporated into the nucleosome core complex is a poor template for transcription by RNA polymerase with the *E. coli* enzyme as a model system (Williamson & Felsenfeld, 1978; Waslyk et al., 1979). Representative data are given in Table II which show that the DNA in the reconstituted nucleosomes is transcribed by *E. coli* RNA polymerases less than 10% as efficiently as naked calf thymus DNA. Williamson & Felsenfeld (1978) have recently shown that 0.3 M salt can enhance transcription of nucleosomal DNA. An ~4-fold increase in transcription of the DNA in these reconstituted nucleosomes is observed on increasing the KCl concentration to 0.3 M (Table II).

Addition of D-55 to the nucleosomes causes a dramatic ~100-fold increase in the efficiency of transcription of the

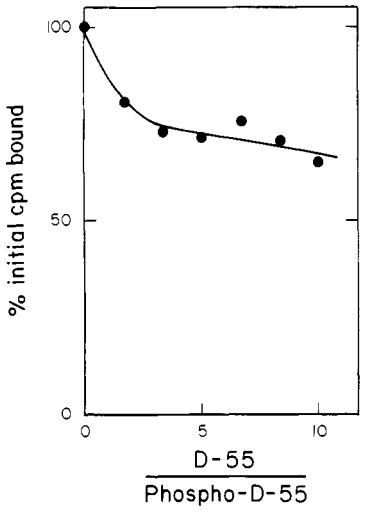

FIGURE 9: Competition between phospho-D-55 and D-55 for binding to nucleosomes. Reconstituted nucleosomes were incubated in 0.67 mL of buffer F containing an excess of [^{32}P]phospho-D-55 and increasing amounts of D-55. The mole ratio of D-55 to phospho-D-55 in the incubation mixture is given on the abscissa. The nucleohistone was precipitated by addition of 30% poly(ethylene glycol) to a final concentration of 10%. The mixture was centrifuged, the supernatant withdrawn, and the pellet resuspended in 10 mL of scintillation fluid for counting. The percent of [^{32}P]phospho-D-55 remaining with the nucleosome compared to incubation in the absence of D-55 is given on the ordinate.

Table II: Activity of *E. coli* RNA Polymerase Using Calf Thymus DNA, Nucleosomes, Nucleosomes Plus D-55, and Nucleosomes Plus Phospho-D-55 as Templates

template	addition to assay	cpm in RNA after 10 min
blank		115 ± 25
calf thymus DNA[a]	none	4900 ± 600
calf thymus DNA, 0.05 M KCl	D-55	5600 ± 400
nucleosomes	none	380 ± 90
nucleosomes	D-55	24345 ± 3500
nucleosomes	phospho-D-55	650 ± 195
calf thymus DNA, 0.3 M KCl		
nucleosomes	none	1245 ± 218
nucleosomes	D-55	7062 ± 115
nucleosomes	phospho-D-55	1763 ± 95

[a] Total amount of DNA in each reaction was the same in 0.05 and 0.3 M KCl reaction and equivalent to the amount of calf thymus DNA in the first two reactions. The calf thymus DNA used for the reconstituted nucleosomes had an average mass of 10^6 daltons.

nucleosomal DNA by the bacterial RNA polymerase (Table II). This increase is seen either when the nucleosomes were reconstituted in the presence of D-55 or when D-55 was added to the reconstituted nucleosomes. The rate of transcription of nucleosomal DNA by *E. coli* RNA polymerase in the presence of D-55 is 2–4 times the rate with the use of the same amount of uncomplexed calf thymus DNA (Table II). The data in Table II represent reaction mixtures containing approximately the same amount of DNA. Salt, 0.3 M, attenuates the enhancement of transcription induced by D-55, perhaps because salt interferes with the binding of D-55. In the presence of D-55, the pattern of DNA fragments resulting from digestion of the nucleosome with staphylococcal nuclease is unaltered (Figure 8b).

Phosphorylation of D-55 abolishes the ability of the protein to enhance the transcription of the nucleosomal DNA (Table II). In contrast to the unphosphorylated protein, the presence of the phosphorylated protein does alter the DNA digestion pattern produced by staphylococcal nuclease, shifting the predominant DNA fragment from ~170 to ~140 base pairs (Figure 8b).

Discussion

The nucleosome has emerged as a fundamental structural unit found in chromatin. Current models based on compositional (Kornberg, 1977), electron microscopic (Langmore & Wooley, 1975), X-ray diffraction (Finch et al., 1977), and neutron scattering studies (Hjelm et al., 1977; Pardon et al., 1975; Suau et al., 1977) suggest the structure of a mononucleosome to be a flattened disk with superhelical DNA wound around the outside of a core made up of two copies each of histones H2A, H2B, H3, and H4. Mononucleosomes containing ~140 base pairs of DNA are connected to each other in the polynucleosome structure by a "spacer" of ~35 base pairs which can combine with histone H1 in an as yet undefined spacial arrangement (Mathis et al., 1980). While the nucleosome is undoubtedly a basic structural component of chromatin, the structure of chromatin in vivo must be considerably more complex, since the native structure must accommodate a large number of nonhistone chromosomal proteins in addition to these "core" particles. The fact that the DNA in the compact nucleosome structure proves relatively refractory to transcription by either heterologous or homologous RNA polymerases (Williamson & Felsenfeld, 1978; Waslyk et al., 1979) also suggests that at least in a part of the chromosome this fundamental nucleosome structure must be altered. It has been suggested that the folded form of nucleosomes may be absent from exceedingly active genes (Franke et al., 1976; Laird et al., 1976; Woodcock et al., 1976). Recent data on nuclease digestion patterns and electron microscopy have been interpreted, however, to show that nucleosomes are present on both inactive and transcriptionally active chromatin (Chambon, 1977; Felsenfeld, 1978; Sonnenblichler, 1979; Mathis et al., 1980) although in an altered form in the latter case. The nature of the altered or transcriptionally active nucleosome or what induces it is unknown.

The compact form of nucleosomes is present at salt concentrations of 10–100 mM, but it has recently been shown that higher salt concentrations, ~0.3 M, alter this structure without the dissociation of the histones so that the DNA becomes more transcriptionally active as reflected in transcription by the *E. coli* RNA polymerase (Williamson & Felsenfeld, 1978; Waslyk et al., 1979) (see Table II). Likewise, at very low salt concentrations (0.3–3 mM), electric dichroism studies show the structure to partially unfold (Wu et al., 1979). Transcriptional activity on the latter form has not been tested. While local changes in ionic strength might be brought about within the nucleus, it seems most probable that the transformation from transcriptionally inactive to active chromatin in vivo is likely to be associated with complex alterations in DNA–protein interactions, possibly triggered by effector molecules and covalent modifications. It has recently been demonstrated that very gentle chromatin fractionation techniques yield nucleosomes to which are attached a variety of the acidic nonhistone chromosomal proteins (Jackson et al., 1979). Two of the high mobility group, HMG1 and HMG2, are reported to be present in nearly stoichiometric quantities.

While identifying nonhistone chromosomal proteins which alter nucleosome structure in functionally significant ways is clearly a formidable task, we attempted to narrow the selection by examining proteins which bind to both components of the nucleosome, DNA and histones, and in addition undergo phosphorylation, since much circumstantial evidence suggests that alterations in transcriptional activity are associated with

changes in the level of phosphorylation of the chromosomal proteins. D-55 was readily identified by using these criteria (Figures 2–4 and 7). This protein binds to nucleosomes (Figure 9) and brings about a striking enhancement in the ability of the DNA to act as a template for the bacterial RNA polymerase (Table II). Moreover, the D-55 accomplishes this without radically altering the structure of the nucleosome as assayed by the nuclease digestion pattern (Figure 8). Lastly, the functional result of binding is completely abolished by phosphorylation (Table II) associated with much tighter binding to histones (Figure 7) and at least some alteration in nucleosome structure, thus providing a plausible control mechanism.

The opposite effect of phosphorylation on the stimulation of transcription by a DNA binding protein specific for one particular set of genes, i.e., the ribosomal genes from the nucleoli of *Physarum polycephalum*, has recently been described by Kuehn et al. (1979). In *Physarum*, treatment with polyamines brings about the phosphorylation of an acidic nonhistone chromosomal protein of M_r 70 000 which is associated with the ribosomal nucleoprotein (rDNP) complex from the nucleoli and is not ordinarily phosphorylated. The phosphorylated protein stimulates the transcription of the ribosomal genes by the homologous RNA polymerase I $\sim$ 100-fold. This protein in the phosphorylated form also binds very tightly to rDNA. Dephosphorylation of this protein with alkaline phosphatase abolishes the stimulation of transcription as well as its ability to bind to the rDNA, although there is not direct evidence that the transcriptional stimulation and DNA binding are directly related, since the stimulation is of the rDNP complex. It thus appears clear that phosphorylation can significantly alter the binding modes of certain proteins to nucleoprotein complexes. The functional consequences resulting might be either positive or negative, depending on the particular topology of the system.

The histone–Sepharose chromatography shows all the D-55 isolated from the fraction B proteins, eluting from hydroxyapatite at 50 mM NaCl, to behave as the dephospho form (Figure 7). In vivo, D-55 may not be exclusively in the dephospho form, since a band migrating at the same position in the acrylamide gel is also present in the fraction C proteins (Figure 1). The latter fraction, eluting from hydroxyapatite at 300 mM NaCl, contains the more highly phosphorylated proteins, and this band may represent the phosphorylated form of D-55.

The amino acid composition of the S-70 protein described here is similar to but not identical with that reported for the *Physarum* protein and also very similar to another protein of M_r 70 000 isolated from Novikoff hepatoma cells (James et al., 1977). We have not observed phosphorylation of S-70, although it comes from the phosphorylatable fraction. If a specific stimulation of the phosphorylating system is required, S-70 may not phosphorylate under the conditions we employed.

While the present findings demonstrate that with the proper protein one can influence the transcriptional accessibility of nucleosomal DNA, attributing physiological significance to this isolated system must be approached with great caution. D-55 does come from the elution of the 30 000*g* pellet and is expected to contain predominantly nucleoproteins. It is present in significant amounts and is isolated with the group grossly identified as the acidic nonhistone chromosomal proteins and is highly acidic. Its phosphorylation appears to be relatively specifically controlled by a nuclear protein kinase. A typical cellular protein kinase, the type II from beef heart cytosol, fails to phosphorylate D-55. D-55 is also distinguished by its high affinity for histones, a feature that perhaps surprisingly does not appear to be a dominant feature of the acidic nonhistone chromosomal proteins, since relatively few species stick to the histone–Sepharose column. The relatively specific nature of the phosphorylation is also suggested by the introduction of a single phosphate group (Figure 7) and the ability of this modification to alter nucleosome structure (Figure 8) and abolish the initial function change induced by the unphosphorylated protein (Table II).

If this protein is indeed involved in some way in facilitating transcription of chromatin, its occurrence in relatively large amounts, at least 10^5 copies per cell, suggests a rather general role, perhaps facilitating the transcription of large groups of genes, since specific control of single gene expression seems likely to rest with proteins recognizing specific DNA sequences occurring in one or a few copies and hence themselves would be expected to be present in only a few copies per cell. The relationship between the facilitation of transcription by CAP protein and the specific control of prokaryotic operons by unique repressor molecules might be a comparable prototype. Relatively low affinity of such proteins for DNA (Figure 6) might not be unexpected, since the complexes may rearrange rapidly and such high affinity functions as specific DNA sequence recognition or melting of the double strand would be functions of the RNA polymerase and other proteins. It could also be postulated that the phosphorylated form of D-55 is involved in inhibiting gene expression.

Determination of whether this in vitro model has an in vivo counterpart clearly requires much further investigation. D-55 bound to the nucleosome can allow the passage of an RNA polymerase and the transient melting of the superhelical DNA without major alteration in the general topology of the winding of the DNA on the nucleosome, since the spacing of the phosphodiester bonds susceptible to nuclease attack is not altered (Figure 8). Transcription by the bacterial RNA polymerase must represent relatively nonspecific transcription of accessible DNA. While the accessibility of the nucleosomal DNA to an enzyme which melts the double strand has obviously been changed by D-55, extrapolation of this model to postulate direct function of D-55 in transcription by the homologous RNA polymerases of calf thymus cannot be made and requires investigation with the eukaryotic RNA polymerases. More sensitive probes than nuclease digestion patterns for alteration in nucleosome structure induced by D-55 also need to be applied.

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Regulation of Muscle Differentiation: Cloning of Sequences from α-Actin Messenger Ribonucleic Acid†

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ABSTRACT: α-Actin is found exclusively in skeletal muscle tissue and appears to be induced during myogenesis. The mechanism underlying the induction of muscle gene transcription can be studied with a deoxyribonucleic acid (DNA) probe complementary to the α-actin messenger ribonucleic acid (mRNA) sequence. Such a probe was produced by cloning complementary DNA (cDNA) transcribed from a breast muscle RNA preparation enriched for actin mRNA. Double-stranded DNA was inserted into the *Pst*I restriction site of plasmid pBR322, and the resulting hybrid DNA molecules were used to transform *Escherichia coli* RR1. Bacterial colonies were preliminarily screened by hybridization to two different [^{32}P]cDNA preparations, i.e., one of which contained sequences of α-actin mRNA and its major contaminant, while the other contained only the major contaminant sequence. The presence of an actin-specific gene insert was documented in plasmid pAC269 with translation assays. Total muscle mRNA was hybridized to pAC269 DNA–cellulose, and the hybridized message was then eluted and translated in a mRNA-dependent reticulocyte lysate. The mRNA which hybridized to pAC269 directed the translation of a protein of 42 000 M_r which was subsequently identified as actin by electrophoretic mobility (one and two dimensions), deoxyribonuclease I (DNase I) affinity, and cyanylation peptide mapping. Restriction endonuclease and heteroduplex mapping of pAC269 detected a 1.4-kilobase insert which is ~95% of the previously measured length of the actin mRNA. When pAC269 was used as a hybridization probe, it was found that the muscle-specific α-actin sequence had only a 70% homology with the nonmuscle β- and γ-actin sequences. Also a difference of 13 °C was observed in thermal melts between muscle and nonmuscle actin mRNAs hybridized to pAC269. These differences in homology and thermal melting will allow the specific quantitation of actin mRNA content during myogenesis and should also aid in the identification of the α-actin natural gene.

The actins are a family of contractile proteins found in all eukaryotic cells (Garrels & Gibson, 1976; Storti et al., 1976; Goldman et al., 1976). Actin was once thought to be a single highly conserved protein, but multiple forms of actin were later identified (Gruenstein et al., 1975; Elzinga & Lu, 1976). α-Actin is found only in skeletal muscle where it is a major constituent of the contractile apparatus. Five other forms of actin, one from cardiac tissue, two from smooth muscle, and two from nonmuscle cells (β and γ), have been identified (Vandekerchove & Weber, 1978; Elzinger & Lu, 1976).

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