Studies of Nuclear Acidic Proteins

EVIDENCE FOR THEIR PHOSPHORYLATION, TISSUE SPECIFICITY, SELECTIVE BINDING TO DEOXYRIBONUCLEIC ACID, AND STIMULATORY EFFECTS ON TRANSCRIPTION*

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SUMMARY

Methods are described for the extraction, separation, and electrophoretic analysis of a class of acidic nuclear proteins from various tissues of the rat. Such proteins occur in the chromatin and their distribution in diverse nuclear types is tissue specific. Many of the chromosomal acidic proteins are phosphoproteins which incorporate 32P-orthophosphate in vivo with the formation of phosphoserine and phosphothreonine residues. The patterns of phosphorylation of individual nuclear proteins vary from one tissue to another.

Many of the acidic proteins prepared from liver and kidney nuclei of the rat form complexes with rat liver DNA. Binding to the DNA of a closely related species (mouse) also occurs, but to a lesser extent. Little or no binding is observed when rat liver phosphoproteins are added under the same conditions to DNA's from calf thymus, human placenta, dog liver, or bacterial cells (Pneumococcus). Nuclear phosphoproteins stimulate transcription in a cell-free RNA-synthesizing system. Correlations are observed between DNA binding and enhancement of RNA synthesis.

DNA-protein complexes have been separated by density gradient centrifugation after "annealing" has occurred. The nature of the proteins present in the complex depends on the tissue from which the nuclear proteins were isolated.

The experiments to be described deal with the isolation and characterization of a complex set of acidic proteins associated with DNA in the nuclei of animal tissues. Evidence will be presented to show that nuclear acidic protein fractions prepared from various tissues of the rat differ in their composition and patterns of phosphorylation. DNA binding by such proteins is selective, and when complex formation occurs, an enhancement of RNA synthesis in vitro can be shown.

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EXPERIMENTAL PROCEDURE

Materials and Methods

Isotope Administration Procedures and Isolation of Nuclei—Adult male Sprague-Dawley rats, weighing 200 to 250 g, were maintained on Purina chow ad libitum and fasted overnight before injection of radioisotopes. 32P-Orthophosphoric acid (carrier free) (Schwarz BioResearch, Orangeburg, New York), neutralized with 0.2N NaOH and adjusted to 0.9% with NaCl, was injected intraperitoneally at a dosage of 2 mCi/100 g of rat body weight. The animals were killed 90 min later and tissues were removed for isolation of nuclei and preparation of the phosphoprotein fractions.

Liver nuclei were prepared as follows: the livers from two to four normal or adrenalectomized rats were chilled and finely minced with scissors. All subsequent steps in the isolation were performed in the cold. The minced tissue was homogenized in 5 volumes of 0.32 M sucrose-3 mM MgCl₂ with a Teflon-to-glass homogenizer of 55-ml capacity, 110-mm grinding length, 44-mm pestle head length, and a clearance of 0.15 to 0.22 mm (Arthur H. Thomas, Inc., Philadelphia, Pennsylvania), rotating at 1560 rpm for 20 strokes. The homogenate was filtered through double napped flannellette cloth, and cold deionized water was added to the filtrate to reduce the total sucrose concentration to 0.25 M. Aliquots of the homogenate were taken for determination of total DNA and protein content. The remainder was transferred to centrifuge tubes and 0.25 volume of 0.32 M sucrose-3 mM MgCl₂ was pipetted into each tube to form a layer beneath the broken cell suspension. The tubes were centrifuged at 1100 x g for 10 min. The crude nuclear pellet was resuspended in 10 volumes of 2.4 M sucrose-1 mM MgCl₂ and the nuclei were sedimented by centrifugation at 100,000 x g for 1 hour. Aliquots of the nuclear pellet were taken for determination of total DNA and protein content. Of the DNA in the original liver homogenate 52% was recovered in the purified nuclear fraction (Table I).

Isolation of nuclei from kidney, spleen, and other tissues followed the procedure described above for liver nuclei.

In some experiments, chromatin was prepared from the purified liver nuclear fraction by the procedure of Dingman and Sporn (1).

Extraction of Nuclear Proteins—Differential extractions were employed to separate the 0.14 M NaCl soluble components and
359. DNA-binding Properties of Isolated Nuclear Acidic Proteins

- Distribution of total and 32P-labeled protein and DNA in extracts of rat liver nuclei

| Fraction analyzed     | Volume | DNA | Protein | 32P activity |
|-----------------------|--------|-----|---------|--------------|
| Homogenate            | 55 ml  | 20  | 1410 mg | 1.12 × 10^6 |
| Nuclei, sedimented thr 2.4 M sucrose* | 18 | 13.5 | 57.62 mg | 1.17 × 10^6 |
| Nuclear extracts      |        | 40  | 11.6 mg | 2.10 × 10^6 |
| 0.14 M NaCl           |        | 40  | 20.8 mg | 1.20 × 10^6 |
| 0.25 M HCl            |        | 15  | 8.49 mg | 1.00 × 10^6 |
| First chloroform-methanol-HCl extract | 10 | 5.66 mg | 1.00 × 10^6 |
| Second chloroform-methanol-HCl extract | 10 | 2.98 mg | 1.00 × 10^6 |
| Ether extract         |        | 1.2 | 6.73 mg | 1.00 × 10^6 |

- Yield of nuclei, based on DNA recovery = 52%. Ratio of total nuclear protein to DNA = 4.2:1.0.
- Ratio of phenol-soluble acidic proteins to DNA = 0.45:1.0.

Histones from the more acidic proteins of the isolated nuclei. The purified nucleolar pellets were suspended in 25 volumes of 0.14 M NaCl and the suspension was centrifuged at 3000 × g for 5 min. The extraction was repeated, and aliquots of the combined supernatants were taken for measurement of the 0.14 M NaCl-soluble nuclear proteins (Table I). Histones and other basic proteins were extracted from the nuclear residue by resuspension in 25 volumes of 0.25 M HCl. After centrifugation at 3000 × g for 5 min, the extraction was repeated. The supernatant phases were combined and analyzed for total histone content (Table I). The nuclear residue was next suspended in 10 volumes of 1:1 (v/v) chloroform-methanol containing 0.2 M HCl and centrifuged at 3000 × g for 5 min. The sediment was extracted with 10 volumes of 2:1 chloroform-methanol containing 0.2 M HCl and centrifuged as before. The pellet was subsequently washed with 10 volumes of ether.

Preparation of Phosphoproteins—The isolation of the nuclear acidic proteins is based on their solubility in phenol. The procedure employed is a modification of that used by Viguéa, Algranati, and Ochoa (2) for isolation of the proteins of the bacteriophage MS 2. The residue remaining after extraction of the 0.14 M NaCl-soluble proteins, histones, and lipids was re-suspended in 5 volumes of 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol (Buffer A). The mixture was mixed gently with an equal volume of cold phenol saturated with Buffer A and allowed to stand for 14 hours at 2°C. The suspension was homogenized briefly and centrifuged at 12,000 × g for 10 min. The aqueous phase was collected, re-extracted with an equal volume of phenol, and centrifuged as before. The combined phenol extracts were dialyzed against 100 volumes of 0.1 M acetic acid containing 0.14 M 2-mercaptoethanol for 3 hours. The dialysis was continued against fresh 0.1 M acetic acid-0.14 M 2-mercaptoethanol until the phenol phase was reduced to one-fifth of its original volume. The dialysis tubing was then opened and the aqueous layer above the phenol phase was removed. The remaining phenol was dialyzed against 0.05 M acetic acid-9.0 M urea-0.14 M 2-mercaptoethanol for 24 hours. Dialysis was continued for 2 hours in 0.1 M Tris-HCl, pH 8.4, containing 8.6 M urea, 0.01 M EDTA, and 0.14 M 2-mercaptoethanol.

This dialysis procedure effectively restores the phenol-soluble proteins to the aqueous phase for further characterization. No precipitation or turbidity occurs under these conditions. Aliquots of the solution were analyzed for total protein content (Table I), for alkali-labile phosphate and phosphoserine, for average amino acid composition, and for contamination by phenol. At this stage, the residual phenol content is less than 0.9 µg of phenol per 100 µg of protein; its concentration is below 0.0001%.

The procedure used to prepare the DNA-binding experiments. The separation and characterization of individual proteins was achieved by polyacrylamide gel electrophoresis.

Analytical Gel Electrophoresis—The nuclear acidic protein fraction constitutes a complex mixture of proteins which can be resolved, at least in part, by electrophoresis in polyacrylamide gels. The procedure used is based on the separation of protein-sodium dodecyl sulfate complexes (3, 4) under conditions in which the distance of migration of individual proteins can be correlated with their molecular weights (4-6). The acidic protein fraction, prepared as described, was dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS and 0.14 M 2-mercaptoethanol for 24 hours. The solution was clarified by centrifugation at 25,000 × g for 15 min. The slight sediment, negligible in amount, was discarded.

Analytical gels were composed of 10% acrylamide, 0.2% N,N'-methylene bisacrylamide, dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS. The solution was filtered and de aerated before use. Polymerization was accomplished by the addition of 0.3 ml of 10% ammonium persulfate (w/v) and 0.03 ml of N,N,N',N'-tetramethylhexamethylenediamine to 40 ml of the acrylamide solution. Gels 9 cm in length were allowed to form in 12-cm Pyrex glass tubes of 6-mm internal diameter. After a 30-minute polymerization period, the gels were previously run at 4.5 volts per cm for 30 min with 0.1 M sodium phosphate buffer, pH 7.4-0.1% SDS in both upper and lower electrode chambers. Samples were then applied to the gels. Each contained approximately 220 µg of the nuclear acidic protein fraction in 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.4, 0.1% SDS, containing 10% (w/v) sucrose and 0.03 ml of tracking dye. Electrophoresis was carried out at 4.5 volts per cm for 7 hours. The gels were removed from the tubes and stained in a solution of Amido black 10B in water-methanol-acetic acid (6:3:1, v/v/v) for 30 min. After destaining in water-methanol-acetic acid (6:3:1), the gels were photographed and then frozen with a block of solid CO₂. The frozen gels were sliced transversely into 1-mm slices, each of which was analyzed for 32P activity and protein content. Quantitative determination of protein in individual bands was carried out in two ways; by densitometry and computer analysis of the densitometer tracing, and by measurements of the dye eluted from the gel slices in dimethylsulfoxide (7). Densitometer tracings were obtained by scanning the negative of the gel photograph with a Joyce-Loebl MK IIIe double beam recording microdensitometer. For determination of radioactivity each slice of the gel was counted.

The abbreviations used are: SDS, sodium dodecyl sulfate; cyclic AMP, adenosine cyclic 3',5'-monophosphate.

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stained) was dissolved in 0.5 ml of NCS solubilizer (Amersham-Searle, Inc., Des Plaines, Illinois) and 15 ml of toluene containing 4.2% Liquifluor (New England Nuclear, Inc., Boston, Massachusetts) was added for counting. 32P activity was measured in a Nuclear Chicago Mark I scintillation spectrometer.

Method of Analysis—DNA was determined by the Dische diphenylamine reaction (8). Protein was measured by the method of Lowry et al. (9). The alkali-labile phosphate content of the phosphoprotein fraction was determined after dialysis to remove urea (which interferes in the colorimetric determination of the phosphomolybdate complex). Aliquots containing 1 to 2 μg of phosphoprotein were dialyzed overnight against 0.1 M Tris-HCl, pH 8.4-0.01 M EDTA-0.14 M 2-mercaptoethanol, and the protein precipitated with 10% trichloroacetic acid. The precipitate was dissolved in 2 ml of 1.0 M NaOH and heated at 100° for 15 min. Inorganic phosphate released into the supernatant was analyzed as the phosphomolybdate complex after acidification with 0.1 ml of 4 N HCl-1 N H2SO4 and precipitation of the protein with 0.1 ml of 0.1 M silicotungstic acid in 0.1 N H2SO4. To the clear supernatant was added 1.5 ml of 5% ammonium molybdate in 4 N H2SO4. The phosphomolybdate complex was extracted in 2.5 ml of 1:1 isobutyl alcohol-benzene, reduced with SnCl2 and measured at 660 μμ (10).

The method of Schaffer, May, and Summerson (11) was used to identify the phosphorylated amino acids. The protein fractions were hydrolyzed in 2 N HCl at 110° for 10 hours and the hydrolysate was chromatographed on Dowex 50 in 0.05 N HCl to separate inorganic phosphate, phosphoserine, and phosphothreonine.

The amino acid compositions of the phosphoprotein fractions were determined by ion exchange chromatography after the method of Spackman, Stein, and Moore (12). The Beckman amino acid analyzer 120B was modified for a 7-fold increase in sensitivity by insertion of a Honeywell expanded range card.

The phenol content of the protein fractions was measured by the 4-aminoantipyrine method of Oehlynski (13).

DNA-binding Studies—For studies of the interaction between phosphoproteins and DNA's from different sources, the 32P-labeled phosphoprotein fraction was prepared by the standard phenol procedure, avoiding the later steps involving treatment with SDS. After dialysis for 2 hours against 0.1 M Tris-HCl, pH 8.4, containing 8.0 μg urea, 0.01 M EDTA, and 0.14 M 2-mercaptoethanol, the protein solution was dialyzed overnight at room temperature against 2 M NaCl-5 μM urea-0.01 M Tris-HCl, pH 8.0. DNA's were pre pared from nuclei isolated from human placenta and from the liver of rat, mouse, dog, cat, chicken, and duck. In all cases, the nuclei were isolated in 2.4 M sucrose-1 mM MgCl2 and washed twice with 0.14 M NaCl. They were lysed in 0.15 M NaCl containing 0.1 μg EDTA and 2% SDS, pH 8.0, and sodium perchlorate was added to a final concentration of 1 M. After deproteinization with chloroform-octanol, the DNA was precipitated with ethanol. It was redissolved in 0.015 M NaCl-0.0015 M sodium citrate, pH 7.0, and digested with pancreatic ribonuclease (previous ly heated at 60° for 15 min to inactivate DNase) at a concentration of 50 μg per ml for 30 min at 37°. Promase was then added (after autodigestion at 37° for 2 hours) to a final concentration of 50 μg per ml and incubation was continued for 2 hours. The solution was deproteinized with chloroform-octanol, and the DNA precipitated with 2 volumes of ethanol. The precipitate was re-dissolved in 0.015 M NaCl-0.0015 M sodium citrate. One-tenth volume of 3 M sodium acetate-1 mM EDTA, pH 7.0, was added with rapid stirring, followed by 0.6 volume of isopropl alcohol. The fibrous DNA precipitate was wound around a glass rod, transferred, redissolved, and reprecipitated as before. The final DNA preparations were washed with 70% ethanol and 90% ethanol, and redissolved in 0.015 M NaCl-0.0015 M sodium citrate, pH 7.0. Calf thymus DNA (Worthington Biochemical Corporation, Freehold, New Jersey) and pneumococcal DNA were individually dissolved in 0.015 M NaCl-0.0015 M sodium citrate, pH 7.0. The latter preparations contained a small amount of contaminating protein (about 1.0% in the calf thymus DNA, and 1.7% in the pneumococcal DNA). All other DNA's tested did not contain detectable amounts of protein. Each DNA solution was dialyzed overnight against 2 μl NaCl-5 μM urea-0.01 M Tris-HCl, pH 8. DNA and protein solutions were mixed under conditions previously employed for the study of the reconstitution of chromatin (15). Approximately 100 μg of protein and 200 μg of DNA were present in a final volume of 0.5 ml of 2 M NaCl-5 μM urea-0.01 M Tris-HCl, pH 8.0. The salt concentration of the mixture was then progressively lowered by stepwise dialysis against 1, 0.8, and 0.6 μM NaCl, allowing 2 hours for each step, and against 0.4 μM NaCl overnight, all in the presence of 5 μM urea. The urea was removed and the salt concentration was lowered by dialysis against 0.01 M Tris-HCl, pH 8.0-0.01 M NaCl for 2 hours. After dialysis, the sample, 0.5 ml, was carefully layered on top of a 4-ml 5 to 25% sucrose gradient in 0.01 M Tris-HCl, pH 8.0-0.01 M NaCl. The tubes were centrifuged at 350,000 × g for 24 hours. They were punctured at the base and 0.2-ml fractions were collected. Each fraction was diluted to 0.4 ml and its optical density at 260 μμ was determined as a measure of its DNA content. The distribution of 32P labeled protein in the gradient was determined by adding 10 μl of Bray’s solution (16) to each fraction and measuring radioactivity by scintillation spectrometry.

In some experiments, the phosphoprotein fraction was treated with ribonuclease in an attempt to determine whether binding to DNA could be influenced by residual (but nondetectable) amounts of contaminating chromosomal RNA's (15). Rat liver phosphoprotein (117 μg in 0.5 ml of 2 M NaCl-5 μM urea-0.01 M Tris-HCl, pH 8.0) was treated with 10 μg of pancreatic ribonuclease (Worthington) which had been previously heated at 60° for 15 min to remove any residual deoxyribonuclease activity. After incubation for 2 hours at 37°, the phosphoprotein fraction was “annealed” to 200 μg of rat liver DNA, subjected to gradient dialysis, and fractionated by sucrose density gradient centrifugation, as described. The peak containing the nucleoprotein complex was isolated and the ratio of protein to DNA was determined. In the RNase-treated samples, the protein to DNA ratio was 0.114 (13.2 μg of protein per 115 μg of DNA), while the corresponding figure for control proteins incubated and annealed in the absence of ribonuclease was 0.117, indicating that enzymatic digestion had little effect on the protein DNA binding reaction. The formation of phosphoprotein-DNA
complexes in the presence of RNase, which is a basic protein, also indicates that this amount of enzyme did not block all the reactive regions of the DNA. Other experiments established that ribonuclease treatment did not remove ³⁵P activity from the isolated nuclear phosphoprotein fraction after labeling in vitro with ³²P-orthophosphate, since no counts were lost when 117 µg of liver nuclear acidic protein was incubated with 20 µg of RNase for 2 hours at 37°C. Parallel experiments were carried out to assure that ribonuclease is functional in the 2 M NaCl-5 M urea-0.01 M Tris-HCl buffer employed in the first stage of the binding experiments. Total rat liver RNA, labeled in vivo with ³⁵S-uridine and isolated by the phenol procedure, was incubated with 50 µg of RNase under the conditions described. Over 88% of the counts were lost in a 2-hour incubation, a result fully equivalent to that observed when the digestion was carried out in 0.1 M phosphate buffer at pH 8.0. It follows that the absence of a ribonuclease effect on the binding reaction is not caused by a loss of activity of the enzyme in the high ionic strength urea buffer.

RNA Polymerase Assay—The effects of nuclear phosphoproteins on transcription were tested in vitro, with the RNA polymerase assay as described by Burgess (17) and comparing the template activities of free DNA and DNA-protein complexes. Assay mixtures of 0.1 ml final volume, containing 0.04 M Tris-HCl buffer, pH 7.9, 0.01 M MgCl₂, 0.10 mM EDTA, 0.10 mM dithiothreitol, 0.15 M KCl, 0.15 mM GTP, 0.15 mM ATP, 0.15 mM CTP, 0.15 mM ³H-UTP (specific activity: 10⁶ cpm per µmole), and 4 to 5 µg (about 0.76 enzyme unit) of Escherichia coli RNA polymerase prepared according to Burgess (17) were "primed" with 2 to 4 µg of free DNA or with the equivalent amount of DNA-protein complex. In some experiments the phosphoprotein-DNA complex was isolated by sucrose density gradient centrifugation as described for the DNA-binding experiments. In other tests, the entire protein-DNA mixture was tested after the annealing and dialysis steps but prior to centrifugation. Triplicate samples were incubated for 10 min at 37°C. The tubes were then chilled in ice and 1.0 ml of DNA carrier solution (10 µg of DNA per ml) was added, followed by 5 ml of 3% trichloroacetic acid containing 0.01 M sodium pyrophosphate. After 30 min the precipitates were collected on Millipore filters (0.45 µm pore size) and washed with 25 ml of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The filters were dried and transferred to counting vials containing 5 ml of toluene-Liquifluor (New England Nuclear, Inc., Boston, Massachusetts). ³H-activity was measured on a Nuclear Chicago Mark I liquid scintillation spectrometer.

RESULTS

Isolation and Properties of Nuclear Acidic Proteins—A new method for the preparation of nuclear acidic proteins, many of which have an affinity for rat DNA, has been applied to the liver, kidney, and other tissues of the rat. The isolation procedure is based on the nuclear localization and characteristic solubility properties of these proteins, which were extracted from nuclei that had been purified by centrifugation through sucrose density barriers to remove whole cells and cytoplasmic contamination. A differential extraction of nuclear proteins was then carried out, with 0.14 M NaCl to remove 0.14 M NaCl-soluble components, 0.25 M HCl to remove histones, and phenol to solubilize a class of nuclear phosphoproteins which show strong indications of involvement in the control of chromosomal RNA synthesis.

The use of phenol to solubilize proteins associated with nucleic acids has precedents in the study of plant viruses (18) and bacteriophages (2), and phenol has been used previously to extract proteins with high rates of synthesis from the nuclei of mammalian cells (19, 20). The present procedure combines phenol extraction with electrophoretic separations of individual proteins from different nuclear types. It permits the demonstration that many of the acidic proteins of the nuclei are tissue-specific phosphoproteins. Some of these combine selectively with DNA and stimulate transcription in an in vitro RNA-synthesizing system.

Localization—The phenol-soluble proteins occur in the nucleus and comprise a qualitatively important fraction of the total nuclear protein. In liver nuclei, for example, where the ratio of total protein to DNA is 4.2 µg per protein of mg of DNA, the phenol-soluble protein fraction is about 12% of the total protein, and the ratio of phenol-soluble proteins to DNA is at least 0.45:1.0 (Table I). Thus, in this metabolically active tissue, there is nearly half as much acidic nuclear protein as there is DNA. The proteins under consideration appear to be components of the chromosomes rather than the nuclear sap. This is indicated by the fact that the proteins remain associated with the DNA during the isolation of chromatin. Electrophoretic analyses of the acidic proteins prepared from liver chromatin show close resemblances to the corresponding fractions prepared from intact nuclei (Fig. 1).

Amino Acid Composition—Amino acid analyses of the phenol-soluble protein fractions of liver and kidney nuclei clearly indicate their acidic nature, i.e. the content of aspartic plus glutamic acids greatly exceeds that of the basic amino acids, lysine, arginine, and histidine (Table II). Although the extent to which the acidic amino acids occur in the polypeptide chains as free acids or as the corresponding amides is not known, the electrophoretic mobility of the proteins at neutral pH values indicates that they have isoelectric points well below pH 7.4. Electrophoresis of ³²P-labeled nuclear phosphoproteins in pH gradients which permit isoelectric focusing reveals that most of the radioactive protein bands occur in regions of the gradient with pH values below 7.0.

Heterogeneity and Tissue Specificity—The phenol-soluble protein fraction is complex. It comprises a mixture of proteins which differ in size, electrophoretic mobility, degree of phosphorylation, and affinity for DNA. The mixture has been resolved into many of its components by electrophoresis in polyacrylamide gels. Electrophoresis in the presence of sodium dodecyl sulfate separates the proteins on the basis of molecular size (2-6). Subsequent staining with Amido black 10R reveals the presence of many proteins with a broad range of molecular weights (Fig. 2). Judging by the mobilities of individual bands, as compared with the mobilities of ³²P-labeled marker proteins of known molecular weights, most of the liver nuclear acidic proteins have molecular weights above 35,000. A molecular weight range of 13,000 to 80,000 is commonly observed under these conditions (20).

The number of protein bands and their relative intensities are highly reproducible in the phenol-soluble protein fractions pre-
phosphorylated from any given tissue, such as the liver. The electrophoretic pattern is not altered if the acidic protein fraction is re-extracted in phenol and reisolated. Estimates of the relative amounts of individual components can be made by (a) cutting out the bands and extracting the dye from each gel slice in dimethylsulfoxide (7) (the amount of dye recovered from each band is directly proportional to the protein content of that band), and (b) by quantitative densitometry and computer analysis of the densitometer tracing.2,4

The complexity of the phenol-soluble protein fraction of rat liver nuclei is indicated by the multiple banding pattern shown in Fig. 2; the presence of over 26 components is indicated. A densitometer tracing of the gel photograph is aligned with the banding pattern and shown in the upper part of the figure. This curve has been resolved into 38 components by computer analysis.

The banding patterns of the nuclear acidic proteins vary from one tissue to another. This is clearly indicated by a comparison of the stained gels and densitometer tracings of preparations of liver (Fig. 2A) and kidney acidic proteins (Fig. 2B). Smaller differences are evident in electrophoretic analyses of the phenol-soluble proteins of spleen nuclei (Fig. 3.1) and brain nuclei (Fig. 3B). Thus, tissue specificity, which should be one of the characteristics of a class of DNA-associated proteins concerned with the control of transcription in differentiated cells, is observed in the phenol-soluble protein fraction. The tissue specificity of the nuclear acidic proteins is also indicated by differences in their rates of phosphorylation in vivo.

**Phosphorylation**—The presence of phosphate in the acidic nuclear protein fraction has been exhibited in three ways: (a) by analysis of the phenol-soluble proteins for alkali-labile phosphate content, (b) by acid hydrolysis of the protein followed by chromatographic separations of phosphoserine and phosphothreonine, and (c) by measuring the incorporation of 32P-labeled orthophosphate in vivo into individual phosphoproteins of rat liver and kidney nuclei.

Phosphate esterified to serine and threonine residues in the nuclear phosphoproteins was released by a brief hydrolysis in 1 N NaOH at 100°C. The resulting inorganic phosphate was measured by colorimetric analysis of the phosphomolybdate complex after its extraction in isobutyl alcohol-benzene (10). By this test, the average phosphorus content of the phenol-soluble protein fraction is 0.94% by weight, a figure which is to be compared with earlier estimates of 1.14% phosphorus in rat liver nuclear phosphoproteins prepared by a combination of salt extraction and chromatographic purification (21).

The presence of the phosphorylated amino acids, phosphoserine and phosphothreonine, was established by hydrolysis of the 32P-labeled protein from liver nuclei and chromatographic separation of the isotopically labeled amino acid esters, using the method of Schaffer et al. (11) to separate phosphoserine from phosphothreonine and from inorganic phosphate. Phosphoserine is the major phosphorylated amino acid in the phenol-soluble protein fraction. 32P-labeled phosphothreonine is also present.

The incorporation and release of phosphate by nuclear phosphoproteins in vivo is a major aspect of their metabolism (10, 21–29). The present studies verify earlier observations on extensive 32P-orthophosphate uptake into nuclear acidic proteins, and extend them to a consideration of 32P incorporation into individual proteins of liver and kidney nuclei. Rats were injected with 32P-orthophosphate and the nuclei of liver and kidney were isolated 90 min later. The phenol-soluble protein

![Fig. 1. Comparison of electrophoretic banding patterns of acidic proteins extracted from intact rat liver nuclei and from isolated rat liver chromatin. SDS complexes of the phenol-soluble proteins were fractionated by electrophoresis in 10% polyacrylamide gels at pH 7.4, as described under "Materials and Methods." The gels were stained with Amido black 10B to indicate the positions and relative concentrations of individual protein bands. The similarities in banding patterns indicate a chromosomal origin for many of the proteins in the whole nuclear extract.](image)

### Table II

**Amino acid composition of phenol-soluble protein fractions from rat liver and kidney nuclei**

| Amino acids   | Source of nuclear acidic proteins |
|--------------|----------------------------------|
|              | Liver | Kidney |
|              | moles/100 moles total amino acid |        |
| Lysine       | 6.04  | 6.72   |
| Histidine    | 2.52  | 2.41   |
| Arginine     | 5.89  | 7.06   |
| Aspartic acid| 10.28 | 9.55   |
| Threonine    | 3.81  | 4.91   |
| Serine       | 4.38  | 5.33   |
| Glutamic acid| 12.16 | 12.29  |
| Proline      | 6.49  | 5.14   |
| Glycine      | 15.28 | 9.62   |
| Alanine      | 6.73  | 7.55   |
| Valine       | 6.10  | 6.31   |
| Methionine   | 1.92  | 2.00   |
| Isoleucine   | 4.13  | 5.22   |
| Leucine      | 6.79  | 8.90   |
| Tyrosine     | 3.26  | 3.23   |
| Phenylalanine| 3.96  | 3.64   |

* Average of three preparations.

Values not corrected for hydrolytic losses.
FIGS. 2, 3 AND 4.
fraction was prepared and separated into its components by polyacrylamide gel electrophoresis. The $^{32}$P activity in each band was measured by slicing the gel transversely and counting individual bands. Clear differences are evident in the extent of phosphorylation of different proteins from the same tissue (Fig. 4A). The pattern of phosphorylation of kidney and liver proteins also appears to be tissue specific (compare Fig. 4, A and B). A complication in such studies is the fact that the rate of phosphorylation of individual proteins in liver nuclei is subject to hormonal regulation. Hydrocortisone, for example, influences both the rate of synthesis (20) and the kinetics of $^{32}$P incorporation into individual proteins of the phenol extract of liver nuclei. In any case, the presence of isotopic phosphate in so many of the nuclear proteins of liver and kidney cells indicates both the extreme complexity and the high metabolic activity of the phosphoprotein fraction.

A number of tests have been carried out to ensure that the $^{32}$P activity observed is not the result of contamination by nucleic acids or phospholipids. When rats were injected with orotic acid-6-14C (15 mCi per rat; specific activity 30.5 mCi per mmole) as a marker for newly synthesized nucleic acids, the nuclear RNA of the liver had a specific activity of over 130,000 cpm per mg after 30 min. The total phosphoprotein fraction had only 20 cpm per mg, and no $^{14}$C activity could be detected in any of the phosphoprotein bands in the polyacrylamide gels. This is in accord with the expectation that contamination by ribonucleic acids would be minimal, because RNA's would be expected to remain in the aqueous phase when the nuclear acidic proteins are extracted in phenol. Similarly, contamination by lipids is minimized by extracting the nuclei with chloroform-methanol-HCl before dissolving the phosphoproteins in phenol. The effective removal of lipids by this procedure was tested by injecting rats with glyceryl-2$^3$H (250 mCi per rat; specific activity 200 mCi per mmole) and measuring incorporation after 3 hours. The total activity in the lipids extracted by chloroform-methanol-HCl was 9900 cpm, while the total activity in the phenol-soluble fraction of liver nuclei was only 3% of that figure. Thus, it is unlikely that incorporation of radioactive phosphate into the phenol-soluble fraction represents contamination of the phosphoproteins by ribonucleic acids or by phospholipids.

Selective DNA-binding by Nuclear Phosphoproteins—There are several indications that interactions between nuclear phosphoproteins and DNA are highly selective. Under the proper conditions, the formation of complexes between phosphoproteins and DNA is surprisingly species dependent. The specificity of the DNA-binding reaction has been studied with preparations of $^{32}$P-labeled phosphoproteins from rat liver and kidney nuclei. The procedure is based on a slow annealing reaction in which samples of DNA and protein, each dissolved in 2 M NaCl-5 M urea-0.01 M Tris-HCl, pH 8.0, were mixed and dialyzed together against a progression of salt solutions of decreasing concentration. After removal of the urea by dialysis against 0.01 M Tris-HCl, pH 8.0-0.01 M NaCl, the samples were layered over a 5 to 25% buffered sucrose gradient and centrifuged. The distribution of DNA in the gradient was determined by its absorption at 260 mp, while the position of the phosphoproteins was indicated by $^{32}$P radioactivity. A binding of proteins to DNA is accompanied by a shift of $^{32}$P activity downward into denser regions of the gradient. Proteins which are not bound to DNA remain in the light, upper portion of the gradient.

The application of this procedure for the separation of DNA-protein complexes is shown for rat liver phosphoproteins and rat liver DNA in Fig. 5A. The binding reaction is dependent upon salt concentration. At low ionic strengths (e.g. 0.01 M NaCl) the cosedimentation of $^{32}$P-labeled phosphoprotein with DNA is evident (Fig. 5A), but binding is not observed at 0.05 M NaCl (Fig. 5B) nor at 0.15 M NaCl (Fig. 5C).

At low ionic strengths, phosphoproteins from rat kidney nuclei also combine with DNA isolated from rat liver (Fig. 6A). This type of association appears to be relatively selective for the DNA of the species from which the phosphoprotein fraction was prepared. No comparable binding of the phosphoproteins of rat kidney nuclei to calf thymus DNA (Fig. 6B) or to pneumococcal DNA (Fig. 6C) was observed. Similar restrictions apply to the binding of rat liver phosphoproteins. Combination with rat DNA is readily indicated (Fig. 5A), but neither calf thymus DNA (Fig. 7A) nor pneumococcal DNA (Fig. 7B) form soluble complexes with the $^{32}$P labeled proteins of rat liver nuclei.

DNA-binding studies have been extended to DNA's prepared from human placenta, and from the livers of the dog, mouse, chicken, and duck. No appreciable binding of rat liver phosphoproteins to human DNA or dog DNA was observed, but binding of rat liver phosphoproteins to mouse DNA does occur. However, the extent of complex formation between the rat nuclear acidic protein and the DNA of this closely related species is less than that seen when both the protein and the DNA come from the rat. For example, the protein to DNA ratio in the peak region of the gradient was 0.11 μg of protein per 1 μg of DNA for rat DNA and 0.08 μg of protein per 1 μg of mouse DNA tested under identical conditions.

The basis for selectivity in phosphoprotein-DNA interactions with the banding pattern in the upper part of each figure. A, rat spleen nuclear phosphoprotein fraction; B, rat brain nuclear phosphoprotein fraction.
FIG. 5. Separation of nuclear phosphoprotein-DNA complexes after reconstitution in vitro. A rat liver nuclear phosphoprotein fraction, labeled in vivo with $^{32}$P-orthophosphate, was "annealed" with rat liver DNA under the conditions described under "Materials and Methods." The salt concentration of the final dialysis step was varied as indicated. The DNA-protein mixture was layered over a 5 to 25% sucrose gradient and centrifuged at 358,000 $\times$ g for 24 hours. The position of DNA in the gradient is indicated by its absorption at 260 nm (---). The distribution of isotopically labeled phosphoprotein is shown by the dashed line. Note that protein binding to DNA, as evidenced by the movement of $^{32}$P activity downward into the denser regions of the gradient, is pronounced at low ionic strengths but not at higher salt concentrations. A, 0.01 M NaCl-0.01 M Tris-HCl, pH 8.0. B, 0.05 M NaCl-0.01 M Tris-HCl, pH 8.0. C, 0.15 M NaCl-0.01 M Tris-HCl, pH 8.0.

FIG. 6. Evidence for selectivity in binding of rat kidney nuclear phosphoproteins to the DNA of the species of origin. The kidney phosphoprotein fraction, labeled in vivo with $^{32}$P-orthophosphate, was isolated and "annealed" with various DNAs as described under "Materials and Methods." Each mixture was layered over a 5 to 25% sucrose gradient and centrifuged at 358,000 $\times$ g for 24 hours. The distribution of DNA is indicated by its absorption at 260 nm (---). The position of the isotopically labeled phosphoprotein is shown by its $^{32}$P activity (---). Note the formation of complexes between rat kidney phosphoproteins and rat liver DNA (A), but not with the DNA of calf thymus (B) or with pneumococcal DNA (C).

It should be noted that the differences in binding efficiency that we have observed are not likely to be the result of differences in mode of preparation or molecular size of the different DNA samples. Dog, human, and rat DNA's were isolated by the same procedure, but only the latter combines with rat nuclear phosphoproteins. Both the rat liver DNA and the pneumococcal DNA used in these studies were found to have a comparable range of sedimentation coefficients, but no binding to the bacterial DNA was observed. We conclude, tentatively, that the binding reaction probably reflects a capacity of the phos-
phosphoprotein to recognize specific nucleotide sequences in the polynucleotide chain. In this respect, the acidic proteins in the phenol-soluble fraction of the mammalian cell nucleus would resemble other proteins for which specific DNA affinities have been shown, such as the lac operon repressor (31, 32), the C1 gene repressor of the bacteriophage λ (33), and RNA polymerase (35).

The question arises as to whether all or only a fraction of the nuclear phosphoproteins combine selectively with DNA in this manner. The problem was investigated by recovering the proteins from the DNA-protein complex. The DNA-protein peak was selected from the gradient and re-extracted with phenol. After the usual isolation procedure, the phenol-soluble proteins were analyzed by polyacrylamide gel electrophoresis. The results are shown in Fig. 8A, which compares the banding pattern of the DNA-bound protein fraction with that of the total phenol-soluble proteins of rat liver nuclei. It is clear that many of the liver nuclear acidic proteins have the capacity to combine with DNA to form a soluble complex under these conditions. The absence of some bands from the DNA-protein complex recovered from the gradient shows that some of the acidic proteins of liver nuclei do not combine as effectively as do others with the DNA of the species of origin. It is not known whether their failure to do so indicates that they have other functions, or whether it reflects changes in molecular configuration of the proteins during the rigorous isolation procedure.

A similar specificity in DNA binding is evident in the analysis of the soluble DNA-protein complex isolated after the annealing of rat kidney nuclear proteins to rat liver DNA (Fig. 8B). In this case, one of the major components of the phenol-soluble fraction (the fastest moving band in the gel electrophoretic pattern) binds to DNA. The stoichiometry of the interaction between nuclear phosphoproteins and the appropriate DNA depends on the conditions of annealing and on the ionic strength during centrifugation. Under the conditions described, the reconstituted acidic protein-DNA complex of rat kidney has a protein to DNA ratio of 0.13:1 (14.9 μg of protein and 115 μg of DNA in the peak region of the gradient). About 13% of the total 32P-labeled protein applied to the sucrose gradient appears in the peak, and 8% of the protein originally added to the annealing mixture is recovered by re-extraction of the DNA-protein complex with phenol. The comparable figures for reconstituted liver phosphoprotein-DNA complexes indicate a protein to DNA ratio of 0.114:1, and binding of 13% of the total protein added. Considering the possibilities for denaturation of the proteins, and the limitations of the reconstitution experiments, it is likely that these figures are deceptively low.

It has been suggested that the regulation of transcription in the cells of higher organisms involves certain low molecular weight chromosomal RNA's which, by combining with histones or other nuclear proteins, permit a specific interaction with the DNA template (e.g. 15, 36, 37). Accordingly, we have tested to see whether the interaction between nuclear phosphoproteins and DNA requires the participation of RNA. To date, the evidence is negative on three counts: (a) no contamination of the nuclear phosphoproteins by radioactive RNA was detected in labeling experiments with orotic acid-6-14C, (b) direct analysis of the phenol-soluble protein fraction by the orcinol reaction showed no detectable amounts of RNA, and (c) the annealing reactions between protein and DNA still took place after treatment of the acidic protein fraction with pancreatic ribonuclease.

For example, the ratio of liver nuclear phosphoprotein to DNA in the peak region of the gradient was 0.114:1 in ribonuclease-treated samples, and 0.117:1 in the corresponding controls. (Parallel experiments confirmed that ribonuclease retains its enzymatic activity during the annealing and dialysis stages of the experiment. Obviously, the presence of small amounts of the enzyme, which is a basic protein, did not block all the acidic protein-combining sites of the appropriate DNA.) Although these results do not rule out the possible presence of very small amounts of nonradioactive RNA which may be shielded from nuclease action, there is no compelling reason to invoke an RNA-DNA hybridization mechanism to account for the selective binding of the nuclear phosphoproteins to the DNA of the species of origin. It seems more probable that the specificity resides in the
Fig. 8. Evidence for the heterogeneity of DNA-binding acidic proteins from rat liver and kidney nuclei. The phenol-soluble fraction was prepared and “annealed” with rat DNA as described under “Materials and Methods.” The DNA-protein complex was separated by sucrose density-gradient centrifugation and the protein bound to DNA was released by extraction in phenol. They were reisolated and examined by electrophoresis in 10% polyacrylamide gels at pH 7.4. The banding patterns and the corresponding densitometer tracings for total phosphoproteins and DNA-bound phosphoproteins are compared. A, rat liver phosphoprotein; B, rat kidney phosphoprotein.

Effects on Transcription—The binding studies show that many of the acidic proteins of rat liver and kidney nuclei have the capacity to combine with DNA in vitro, and that this combination is selective with respect to both the DNA and the protein components of the complex. Evidence that phosphoproteins are closely associated with DNA in vitro is provided by the presence of many of the proteins in isolated chromatin (Fig. 1), and by autoradiographic studies by Benjamin and Goodman (38) which show a localization of 32P-labeled proteins along the salivary gland chromosomes of Sciona larvae.

The functional significance of the binding of acidic nuclear proteins to DNA has been investigated in a cell-free RNA-synthesizing system. Reaction mixtures containing phosphoproteins from rat liver or rat kidney nuclei and DNA’s from different sources were incubated in the presence of the four ribonucleoside triphosphates and the soluble RNA polymerase enzyme of E. coli (17). The utilization of 3H-UTP was taken as a measure of RNA synthesis. The results, summarized in Table III, show that isotope incorporation into RNA is stimulated when phosphoprotein fractions are added to rat DNA. Higher template activities are particularly evident in the phosphoprotein-DNA complexes isolated by sucrose density gradient centrifugation. For example, RNA synthesis directed by the soluble acidic protein-DNA complex of the liver is 78 to 90% higher than that observed with rat liver DNA alone. Similarly, the kidney acidic protein-DNA complex is 72% more active in transcription than is the corresponding amount of free rat DNA. The stimulation of RNA synthesis by nuclear acidic proteins is also observed in unfractionated mixtures of the phenol-soluble proteins and rat DNA, but the increase is less than that seen with gradient-isolated complexes (Table III). This suggests that some components of the mixture may act to suppress transcription.

An important point is that stimulation of transcription does not occur for all DNA’s. Mixtures of rat liver nuclear phosphoproteins and calf thymus DNA are not more active than thymus DNA alone (Table III). These results support the DNA-binding studies shown in Fig. 6B and 7A which indicate that soluble nucleoprotein complexes are not formed when rat nuclear acidic proteins are added to bovine DNA. Calf thymus DNA recovered from the peak region of the gradient after admixture with rat nuclear proteins is not a better template for RNA synthesis than the original DNA preparation (Table III). Thus, the interactions between nuclear acidic proteins and DNA’s are selective. When complex formation occurs, as it does for rat nuclear proteins and rat DNA, transcription is enhanced. When complex formation fails to occur, as in the case of calf thymus DNA and rat liver phosphoproteins, RNA
TABLE III
Selective stimulation of DNA-directed RNA synthesis by nuclear acidic proteins

RNA polymerase assay was carried out as described under "Materials and Methods," with free DNA's, DNA-protein mixtures, and DNA-protein complexes isolated by sucrose density gradient centrifugation.

| Conditions of experiment | 3H-UTP Utilization for RNA synthesis |
|--------------------------|-------------------------------------|
|                          | Enzyme concentration | Experiment 1 | Experiment 2 |
|                          | units/0.1 ml | cpm/μg DNA | % | cpm/μg DNA | % |
| Rat liver DNA           | 0.75       | 440       | 100 | 965       | 100 |
| Rat liver DNA + rat liver acidic proteins: unfractionated mixture | 0.75 | 697 | 158 |
| Rat liver DNA + rat liver acidic proteins: isolated complex | 0.75 | 835 | 150 | 178 |
| Rat liver DNA + rat kidney acidic proteins: unfractionated mixture | 0.75 | 597 | 136 |
| Rat liver DNA + rat kidney acidic proteins: isolated complex | 0.75 | 734 | 172 |
| Calf thymus DNA         | 1.5        | 2180      | 100 | 5000      | 100 |
| Calf thymus DNA + rat liver acidic proteins: unfractionated mixture | 1.5 | 5600 | 112 |
| Calf thymus DNA + rat liver acidic proteins: isolated peak area of gradient | 1.5 | 2200 | 103 | 4700 | 94 |

Synthesis is not enhanced. The results are in accord with the view that the acidic nuclear protein fraction includes components that facilitate the transcription of the appropriate nucleotide sequences of the corresponding genome.

DISCUSSION

Interest in the nonhistone proteins associated with DNA in the chromosomes of higher organisms stems from observations on their metabolism, localization, and effects on transcription.

Unlike histones, the acidic proteins of nondividing cell nuclei have high rates of synthesis and turnover (19, 39-49). The synthesis of certain nuclear protein fractions is selectively influenced by hormones, such as hydrocortisone and estradiol, which stimulate RNA synthesis in the target tissues (20, 34). The localization of the nonhistone proteins suggests a role in transcription. They are present in high concentrations in the chromatin of metabolically active tissues (1, 30-34), and they are preferentially localized in those regions of the chromatin that are most active in RNA synthesis (21, 55, 56). Since many of the acidic proteins of the nucleas are phosphoproteins (10, 21-29) whose rates of phosphorylation are increased at times of gene activation (29) and which are relatively deficient in cell types that are not actively engaged in RNA synthesis (57), one may surmise that such proteins are likely to be involved in positive control mechanisms, rather than in the suppression of genetic activity.

Evidence that acidic nuclear proteins may counteract the inhibition of RNA synthesis by histones has been obtained repeatedly in many laboratories (e.g., 21, 24, 55, 58-60). Studies of RNA synthesis by reconstituted chromatin fractions also indicate that selectivity in transcription is influenced by the nonhistone proteins added to the mixture (91, 92). Our present findings show that the acidic protein fraction includes tissue-specific components which are highly selective in their interactions with DNA and which increase rates of RNA synthesis from the appropriate DNA templates. We propose that their function is to regulate transcription.

The differentiated cells of higher organisms synthesize different populations of messenger RNA molecules, and they would be expected to differ in their contents of proteins which control this aspect of chromosomal activity. The varied electrophoretic patterns of the phenol-soluble nuclear proteins from different tissues indicate that each differentiated cell type does contain a specific population of DNA-associated nonhistone proteins. Differences exist both in the nature and the relative concentrations of individual protein bands in the acrylamide gel pattern (63).

On the other hand, all cells synthesize certain RNA's in common, such as ribosomal, 5S, and aminoacyl transfer RNA's, and one would expect to find a broad distribution of proteins concerned with the transcription of the more ubiquitous RNA types. Comparisons of the acrylamide gel patterns indicate that similar phosphoproteins exist in diverse nuclear types. The similarities in nonhistone chromosomal proteins have been noted and emphasized by other investigators (e.g. 64). They can be interpreted in terms of common structural proteins and enzymes concerned with nucleic acid biosynthesis and histone metabolism. It is not yet known whether any of the components of the phenol-soluble nuclear protein fraction are enzymatically active in RNA synthesis or RNA methylation, or in the modification of histone structure by acetylation, methylation, or phosphorylation. Clarification of this problem will require isolation and tests for function of individual components of the acidic nuclear protein fraction (assuming that enzymatic activity will not be irreversibly lost during the isolation procedure). The isolation and properties of two nuclear proteins, one from liver, and one from kidney, will be described shortly.

A major aspect of the metabolism of many of the nuclear acidic proteins is the incorporation of 32P-orthophosphate into phosphoserine and phosphothreonine residues in the polypeptide chains. ATP is the phosphoryl group donor in this esterification reaction (10, 21, 27). The phosphorylation is enzymatic, it occurs in the nucleus, and it takes place after protein synthesis has occurred; i.e. 32P-phosphate uptake into nuclear phosphoproteins is not affected when 14C-serine incorporation is completely blocked by puromycin (10). The significance of the phosphorylation of proteins associated with DNA in the chromatin has yet to be determined, but recent studies relate phosphorylation to RNA synthesis. The pattern of phosphorylation of individual acidic proteins of the liver nucleus is altered in a complex way when hormones, such as cortisol, stimulate RNA synthesis in the liver. The changes in phosphate uptake are rapid, specific, and not the result of fluctuations in ATP pool sizes. Together with earlier evidence that nuclear phosphoprotein metabolism is greatly accelerated during gene activation in lymphocytes (29, 65), the present findings in liver and kidney nuclei support the view that phosphorylation of the
DNA-associated proteins in chromatin is somehow related to the specific regulation of RNA synthesis. An important point is that phosphorylation is reversible, and that the turnover of phosphate group turnover on the hydroxylated amino acids, reflects the RNA synthetic capacity of the cell. They combine selectively with the DNA of the appropriate species. Transcription from the DNA-phosphoprotein complex, once formed, proceeds that from DNA alone. We conclude that the nuclear phosphoprotein fraction includes components which are involved in the positive control of RNA synthesis. It is likely that such components recognize and combine with specific nucleotide sequences to promote transcription at particular gene loci. Phosphorylation seems to be a critical variable in the interaction between the phosphoproteins, DNA, and the RNA polymerase. In many respects, the nuclear phosphoprotein control system is analogous to the u factor control of DNA and proteins which regulate transcription in prokaryotic and eukaryotic cells.

Conclusions—The nuclear phosphoproteins have many characteristics of proteins concerned with the specific control of genetic activity in the cells of higher organisms. They are localized in active regions of the chromatin. Their distribution in diverse cell types is tissue specific. Their activity, particularly with regard to phosphate group turnover on the hydroxylated amino acids, reflects the RNA synthetic capacity of the cell. They combine selectively with the DNA of the appropriate species. Transcription from the DNA-phosphoprotein complex, once formed, proceeds that from DNA alone. We conclude that the nuclear phosphoprotein fraction includes components which are involved in the positive control of RNA synthesis. It is likely that such components recognize and combine with specific nucleotide sequences to promote transcription at particular gene loci. Phosphorylation seems to be a critical variable in the interaction between the phosphoproteins, DNA, and the RNA polymerase. In many respects, the nuclear phosphoprotein control system is analogous to the σ factor control of RNA polymerase activity in bacterial systems (66-70). The σ factors appear to confer specificity on the polymerase by facilitating its attachment to specific initiation or “promoter” sites on the DNA. After initiation, the σ factor is released from the enzyme and it can participate in a new round of initiation. Phosphorylation of Escherichia coli σ factors by protein kinases from animal tissues has been found to stimulate RNA synthesis (71). The σ-phosphorylation reaction is itself stimulated by cyclic AMP (71).

The resemblance of the σ-phosphorylation system, particularly with respect to hormonal (cyclic AMP) response, to the phosphorylation mechanisms known to exist for chromosomal proteins in higher cells is strikingly close. Further developments in this area promise to reveal protein phosphorylation as a general mechanism for influencing the interaction between DNA and proteins which regulate transcription in prokaryotic and eukaryotic cells.

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