Rat Embryo Nonhistone Chromosomal Proteins: Interaction in Vitro with Normal and Bromodeoxyuridine-Substituted DNA†

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ABSTRACT: Rat embryo cell nonhistone chromosomal proteins (NHCP) were partially characterized and reacted in vitro with DNA in order to more accurately determine the molecular nature of latent oncornavirus activation by the thymidine analogue 5-bromodeoxyuridine (BrdUrd). Following extraction, purification, and iodination with ^{125}I, the proteins were characterized by amino acid analysis and polyacrylamide gel electrophoresis, and reconstituted with DNA in vitro by affinity chromatography and by gradient dialysis recombination. Radiolabeled NHCP were reacted with homologous and heterologous DNA samples, as well as with DNA extracted from rat embryo cells exposed to a virogenic dose of 5-bromodeoxyuridine. Both modes of protein–nucleic acid recombination revealed a consistent amount of NHCP with an affinity for DNA. Although comparable fractions of NHCP were bound to BrdUrd-treated as well as to untreated rat embryo DNA, incorporation of bromouracil resulted in an increased stability of the complex, as determined by two different techniques. Some rat embryo NHCP demonstrated an affinity for heterologous DNA, although a subpopulation of species-specific NHCP bound differentially to rat DNA by comparison. The composition and molecular weights of total NHCP preparations were highly complex and heterogeneous; however, the DNA-binding subfractions were relatively more uniform. The DNA-binding nature of rat embryo NHCP and their affinity for bromouracil-substituted DNA may be related to the mechanism of type C RNA virus activation from normal rat embryo cells exposed to low doses of the analogue.

It is currently believed that certain nonhistone chromosomal proteins (NHCP)[1] may exert an influential effect in the regulation of expression of the eukaryotic genome. Recent experimental evidence has shown that responsibility for the highly specific control of eukaryotic genetic activity probably does not reside within the histone component of nuclear proteins (Paul and Gilmour, 1968; Stein et al., 1974). Since interaction of DNA-binding proteins with nucleic acids in prokaryotic organisms is essential for regulation of particular gene functions (Dickson et al., 1975), comparable relationships may be important for higher eukaryotes as well. Indeed, many workers have characterized populations of DNA-binding NHCP specific for homologous (Teng et al., 1971) and heterologous (van den Brock et al., 1973) animal cell DNA. Moreover, subclasses of NHCP were recently described which differentially bound to low and high C_0t (Allfrey et al., 1973) and to intermediate C_0t (Sevall et al., 1975) DNA nucleotide sequences. Therefore, the identification of DNA-binding NHCP with such high degrees of specificity and diversity strongly implies equally specific interaction and function with chromosomal DNA in vivo.

The halogenated thymidine analogue 5-bromodeoxyuridine (BrdUrd) has been utilized extensively in many biological systems to study the selective activation or suppression of specific genetic functions (Rutter et al., 1973). In the majority of these experimental systems, substitution for thymine by bromouracil within eukaryotic DNA is an essential prerequisite in order for BrdUrd to exert its manifold differential effects on cell behavior (Rutter et al., 1973; Stellwagen and Tomkins, 1971). Moreover, several investigators recently characterized distinct structural and biophysical alterations to BrdUrd-substituted chromatin (Lapeyre and Bekhor, 1974; Augenlicht et al., 1974; David et al., 1974). It is, hence, reasonable to suggest that the highly specific interactions in vivo between the DNA-binding NHCP and analogue-substituted nucleic acids are sufficiently disrupted to account for the observed modifications in cell behavior.

We previously described the activation and release of an endogenous type C RNA virus from normal rat embryo cell cultures exposed for short periods of time to low doses of BrdUrd (Schwartz et al., 1974). This phenomenon was dependent upon incorporation and substitution of bromouracil in place of thymine into finite regions of rat DNA (Schwartz and Kirsten, 1974). This work was performed in order to further characterize the molecular nature of rat embryo cell NHCP as well as determine their interaction with normal and BrdUrd-substituted rat DNA in vitro. In this manner, the previously documented nucleic acid structural alterations attributable to bromouracil incorporation were correlated with binding and affinity of rat embryo NHCP to purified DNA. These observations may clarify the mechanism(s) by which BrdUrd activates viral gene expression in particular, as well as the regulatory function of the NHCP in general.

Materials and Methods

Cell Cultures. Cells were obtained from a highly inbred colony of Wistar/Furth rats. Fetuses were aseptically removed on the 15th or 16th day of gestation and prepared for growth in vitro in monolayer form as detailed previously (Schwartz, 1976). Resultant cell culture strains were used for NHCP and DNA extractions shortly after initiation in vitro in order to maximize the reproducibility of the results as well as the diploid nature of the cells.

Isolation of Nuclei. Confluent cell cultures were scraped from 32-oz glass bottles with a rubber policeman. The pooled cell suspensions were thoroughly washed several times in phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 1% phenol red) at 5 °C. The cells

† From the Department of Pathology, University of Chicago, Chicago, Illinois 60637. *Received December 13, 1976; revised manuscript received April 26, 1977.* This work was supported by American Cancer Society Research Grant 75-35 and United States Public Health Service Research Grant CA-14898.

[1] Abbreviations used are: NHCP, nonhistone chromosomal proteins; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

were concentrated by low-speed centrifugation and resuspended in 5 volumes of ice-cold 0.32 M sucrose, 3 mM $MgCl_2$. The cells were then homogenized in a Teflon/glass electric homogenizer (Arthur Thomas, Inc.) for approximately 20 strokes at low speed until no large tissue particles could be observed. The homogenate was centrifuged at 5 °C for 10 min at 1500*g*. The resultant pellets were resuspended in 0.25 M sucrose, 3 mM $MgCl_2$, and centrifuged as before. The crude nuclear preparations were similarly washed several more times until the supernatant fractions were clear. The nuclear pellets were then layered onto a 2-mL cushion of 2.0 M sucrose, 1 mM $MgCl_2$, and centrifuged at 100 000*g* for 1 h at 5 °C in a Spinco 50 Ti rotor. The purified nuclei were examined by phase microscopy in order to confirm the absence of subcellular debris.

Extraction of Nuclear Proteins. The phenol solubility technique of Teng et al. (1971) for the purification of NHCP was utilized with some modifications. Briefly, purified rat embryo nuclei were sequentially extracted stepwise at 5 °C in 0.14 M NaCl, 0.25 N HCl, chloroform–methanol (1:1, v/v, in 0.2 N HCl, and 2:1, v/v, in 0.2 N HCl) and finally washed in diethyl ether. The subsequent residue was gently resuspended in 5 volumes of 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol, mixed with an equal volume of cold phenol previously saturated in the same buffer, and allowed to stand for 14 h at 5 °C. The mixture was gently homogenized and centrifuged for 10 min at 12 000*g*, and the phenol phase was retained. This organic fraction was made 0.1 M acetic acid and 0.14 M 2-mercaptoethanol until the phenol phase was reduced to approximately one-fourth of its original volume. The aqueous layer was discarded, and dialysis was continued for 24 h against 0.05 M acetic acid, 9.0 M urea, and 0.14 M 2-mercaptoethanol. After 2 h of dialysis against 0.1 M Tris-HCl, pH 8.4, 8.6 M urea, 0.01 M EDTA, and 0.14 M 2-mercaptoethanol, the proteins were brought to 2 M NaCl, 5 M urea, 0.01 M Tris-HCl, pH 8.0. The solutions were placed in a Spinco 50 Ti rotor and centrifuged for 16 h at 100 000*g* at 5 °C in order to pellet residual DNA. The purified rat embryo NHCP were then utilized for further analytical procedures.

Iodination of Nuclear Proteins. Following ultracentrifugation, protein solutions were stepwise and progressively dialyzed for 2 h against 0.01 M Tris-HCl, pH 8.0, 5 M urea buffers containing 1, 0.8, and 0.6 M NaCl, respectively (Teng et al., 1971). The urea was removed by dialysis against several changes of 0.2 M NaCl, 0.01 M Tris-HCl, pH 8.0, for 24 h, and the protein content was determined according to the method of Lowry et al. (1951). Approximately 350 μg of protein in 1 mL of buffer was reacted with 40 μL of $Na^{125}I$ (2 mCi/mL, New England Nuclear Corp.) and 20 μL of chloramine-T (9 g/mL, Aldrich Chemical Co.) for 20 min on ice (Hunter, 1973). The reaction was stopped with 40 μL of sodium metabisulfite (22.5 mg/mL) (Hunter, 1973) and dialyzed extensively at 5 °C against several changes of 2 M NaCl, 0.01 M Tris-HCl, pH 7.4. Following this procedure, ^{125}I-labeled rat embryo NHCP were stored at 5 °C for several days without appreciable loss of radioactivity or protein integrity.

Analytical Gel Electrophoresis. Aliquots of the radiolabeled NHCP were prepared for characterization by polyacrylamide gel electrophoresis in the manner of Teng et al. (1971). Samples of the ^{125}I-labeled protein fractions were dialyzed at room temperature overnight against 0.01 M sodium phosphate buffer, pH 7.4, made 0.1% in sodium dodecyl sulfate ($NaDodSO_4$), and 0.14 M 2-mercaptoethanol. Gels 9 cm in length were composed of 10% acrylamide, 0.2% *N,N'*-methylenebisacrylamide (Bio-Rad Labs, Inc.) in phosphate buffer, pH 7.4, containing 0.1% $NaDodSO_4$. Gel solutions were polymerized by the addition of 10% ammonium persulfate (w/v, Bio-Rad Labs, Inc.) and *N,N,N',N'*-tetraethylmethylenediamine (Temed, Bio-Rad Labs, Inc.). Following polymerization, gels were placed in a Bio-Rad Model 150A polyacrylamide gel electrophoresis device containing 0.1 M phosphate buffer, pH 7.4, 0.1% $NaDodSO_4$ in both chambers. Samples (0.2 mL) of ^{125}I-labeled rat embryo NHCP were made 12% (w/v) in sucrose, 0.1% in bromophenol blue, and electrophoresed for 8 h at 4.5 V/cm. The gels were removed from the glass tubes and placed overnight in a solution of 0.1% Coomassie blue in water–methanol–acetic acid solution (6:3:1, v/v). After destaining in the same solution, the gels were photographed, scanned at A_{550} in a Gilford 250 spectrophotometer equipped with a linear-transport device, serially sectioned by a mechanical gel-cutting device into 1-mm-thick fractions, and assayed for ^{125}I in a Nuclear Chicago Model 8725 gamma spectrometer.

DNA Labeling and Extraction. To prepare radiolabeled DNA, rat embryo cell cultures in log phase of growth were exposed to either 10^{-7} M [^3H]thymidine (18.3 Ci/mmol, New England Nuclear Corp.) or 10^{-7} M [^3H]BrdUrd (12.7 Ci/mmol, New England Nuclear Corp.) for 24 h. The culture fluids were removed, and the cells were dissolved directly within the 32-oz glass bottles with 10 mL of SSC buffer (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) made 1% in $NaDodSO_4$ and 2-mercaptoethanol (Schwartz, 1976). The lysates were combined, chilled to 5 °C, extracted extensively, and purified as described (Schwartz and Kirsten, 1974). In this manner, unlabeled, [^3H]thymidine-labeled, and [^3H]-BrdUrd-labeled rat embryo DNA samples were prepared from tissue culture sources. Calf thymus and salmon sperm DNAs were obtained from Miles Labs Inc. and were reextracted as above. All purified DNA preparations were relatively free of endogenous protein contamination as determined by A_{260}/A_{280} ratios, and by the method of Lowry et al. (1951).

DNA–NHCP Recombination. Recombinations between ^{125}I-labeled rat embryo NHCP and DNA from various origins were performed in vitro as previously described (Bekhor et al., 1969). Briefly, the respective protein and nucleic acid solutions were made 2 M NaCl, 5 M urea, 0.01 M Tris-HCl, pH 8.0, and 1 mM EDTA. The two molecular components were combined at a protein–DNA ratio of 5:1 (w/w) in a final volume of approximately 0.5 mL. The salt concentration was progressively lowered by stepwise dialysis at 5 °C against 1.0, 0.8, and 0.6 M NaCl for 2 h. The reconstituted mixtures were dialyzed overnight against 0.4 M NaCl, still in the presence of 5 M urea. The salt concentration was further decreased, and the urea removed by dialysis against 0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, and 1 mM EDTA for an additional 16 h. The samples were opened, 0.2 mL was layered onto a linear 5–25% sucrose gradient in the same buffer, and sedimented at 50 000 rpm for 210 min in a Spinco SW-50.1 rotor. Fractions of 0.25 mL were collected and assayed for ^3H and ^{125}I in a Packard Model 3380 scintillation spectrometer and a Nuclear Chicago Model 8725 gamma spectrometer, respectively.

Preparation of DNA–Cellulose Affinity Columns. Nonionic cellulose (Cellex-410) obtained from Bio-Rad Labs, Inc., was prepared according to Alberts and Herrick (1971) for adsorption of DNA. Briefly, the cellulose was treated several times with boiling ethanol to remove pyridine, and then sequentially washed by suspension and filtration in 0.1 M NaOH, 1 mM EDTA, and 10 mM HCl solutions, respectively. This procedure was rapidly performed at room temperature in order to minimize any alterations of cellulose induced by

excessive acidity or alkalinity. The cellulose was then washed thoroughly with distilled, deionized water to neutrality, lyophilized to dryness, and stored in sealed glass vessels. To prepare DNA–cellulose columns, labeled and unlabeled DNA solutions (1–5 mg/mL in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) were mixed with cleaned cellulose at a cellulose/DNA ratio of 1:3 (w/v) in a 100-mm glass Petri dish until a thick, moist paste was formed. The mixture was spread uniformly and allowed to dry at room temperature overnight. The resultant material was then gently ground to a powder and lyophilized another 24 h. The cellulose–DNA was resuspended in absolute ethanol and poured into a 100-mm glass Petri dish to form a thin layer. The open Petri dishes were slowly rocked on a mechanical stage approximately 10 cm from a low-pressure mercury lamp (General Electric, germicidal G15T8, 15 W) for 20 min (Litman, 1968). The irradiated cellulose suspensions were separated by low-speed centrifugation at 5 °C and washed several times in 0.01 M Tris-HCl, 1 mM NaCl, 1 mM EDTA, pH 7.4. The efficiency of DNA adsorbed to cellulose by this procedure was nearly 90%, as determined by the manner of Litman (1968). The cellulose preparations were stored in suspension form at 5 °C with little or no loss of bound DNA after several months.

DNA–Cellulose–NHCP Affinity Chromatography. DNA–cellulose suspensions were poured to a height of nearly 5 cm in a 10 cm × 7 mm glass column (Econocolumn, Bio-Rad Labs, Inc.) at 5 °C. ^{125}I-labeled NHCP brought up in 0.05 M NaCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA were passed through each column at a protein/DNA ratio of 1:1 (w/w), and adjusted to flow at a rate of 4–5 mL/h. Unbound proteins were removed with three successive washes of 2 bed volumes of the same buffer. The remaining DNA-binding rat NHCP were subsequently eluted with buffers of increasing ionic strength until 2.0 M NaCl was reached. Radiolabeled, DNA-binding NHCP were concentrated by dehydration through dialysis tubing (Kleinsmith, 1973), prepared for analytical polyacrylamide gel electrophoresis, electrophoresed, and assayed as described above.

Amino Acid Analysis of NHCP. Small fractions of purified, unlabeled NHCP preparations were hydrolyzed by 6 N HCl at 100 °C for 21 h, and assayed by a Technicon amino acid analyzer. A modified gradient was employed which yielded complete separation of all amino acid (Schwartz and Dorfman, 1975).

Thermal Stability of Reconstituted Complexes. The renatured complexes, as isolated from sucrose gradient centrifugation, were dialyzed overnight at 5 °C against 0.08 M sodium phosphate buffer, pH 6.8 (Schwartz, 1976). The samples were then adsorbed at 60 °C to a thermal hydroxylapatite column equilibrated in the same buffer. The columns were then eluted with 3 bed volumes of 0.18 M phosphate buffer as the temperature was incrementally increased stepwise to 95 °C. A representative aliquot of each eluate was assayed for ^{3}H-labeled DNA as before (Schwartz, 1976).

Results

Rat Embryo NHCP. Normal rat cell cultures were prepared exclusively from 15- or 16-day old Wistar/Furth embryos. Furthermore, only low-passage, diploid rat embryo cells were used for NHCP extraction in order to minimize alterations in nuclear composition resultant from long-term cultivation in vitro. In this manner, rat embryo cell NHCP profiles were consistently similar and reproducible from preparation to preparation. The purified proteins were free of residual DNA as determined by the diphenylamine reaction (Burton, 1956). The partial amino acid analyses of total rat embryo NHCP (Table I) revealed their acidic nature and were comparable to those levels previously reported for phenol-extracted rat kidney and liver NHCP (Teng et al., 1971). For these rat tissue types, glycine, glutamic acid, and aspartic acid altogether accounted for nearly 36% of the total amino acid composition of the NHCP preparations. It should be noted, however, that, since acid hydrolysis does not allow measurement of all amino acids, the aspartic and glutamic acid values are probably elevated.

TABLE I: Amino Acid Analysis of Total Rat Embryo Nonhistone Proteins.[a]

Amino acid	Value
Asp	9.0
Thr	5.4
Ser	8.8
Glu	10.2
Pro	6.8
Gly	14.9
Ala	7.9
Val	5.0
Ile	3.2
Leu	7.2
Tyr	2.3
ϕ Ala	3.1
Lys	6.6
His	2.5
Arg	6.5

[a] Values (the average of three determinations) are expressed as mol/100 mol of total amino acids and are uncorrected for losses from acid hydrolysis.

The conditions for in vitro iodination of proteins by the chloramine-T reaction were selected to achieve high specific activities with minimal proteolysis (Hunter, 1973). Preparations of rabbit immunoglobulin, ribonuclease, and chick embryo xylosyltransferase retained biological specificity and activity when iodinated in the exact manner. Comparable electrophoretic profiles of rat embryo NHCP were always obtained regardless of the chloramine-T reaction, or the presence of inhibitors of proteolysis during extraction of nuclei. It was therefore concluded that conformational, structural, and biological specificities inherent in rat embryo NHCP were not significantly altered during the extraction or iodination procedures.

Analytical Gel Electrophoresis. Following polyacrylamide gel electrophoresis of NHCP preparations, the typical pattern most consistently observed was that of multiple bands of protein with great variation of molecular weights. The corresponding electropherograms derived from A_{550} scans of stained gels similarly disclosed heterogeneity of both abundance and molecular composition of the NHCP species (Figure 1). The molecular weights as resolved by polyacrylamide gel electrophoresis ranged approximately from 10 000 to 140 000 when compared to several calibration protein standards electrophoresed under identical conditions. The distribution of ^{125}I in sectioned gels closely corresponded to the A_{550} absorbance scans (Figure 2). Those individual NHCP species in greatest abundance, as determined by absorbance, proportionately contained the greatest amount of radioactivity. These findings suggested that the total radiolabeled NHCP were uniformly and randomly substituted with ^{125}I.

DNA–Cellulose Affinity Chromatography. DNA extracted and purified from rat embryo cell cultures was consistently free of residual protein. On the other hand, the commercially obtained DNA samples were frequently contaminated with protein. In those instances, the DNA was reextracted and precipitated with ethanol. The molecular weights of the puri-

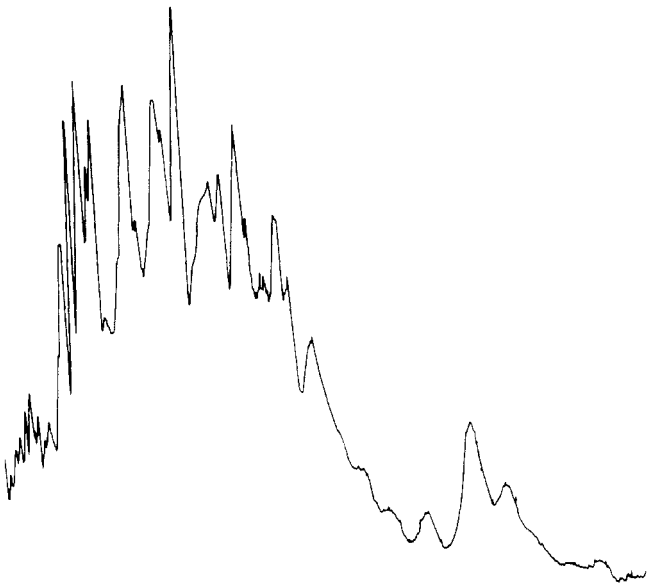

FIGURE 1: Electropherogram of a typical preparation of total rat embryo cell nonhistone chromosomal protein population. Gels were stained with Coomassie blue and scanned at A_{550} as described under Materials and Methods. Direction of electrophoretic migration is from left to right.

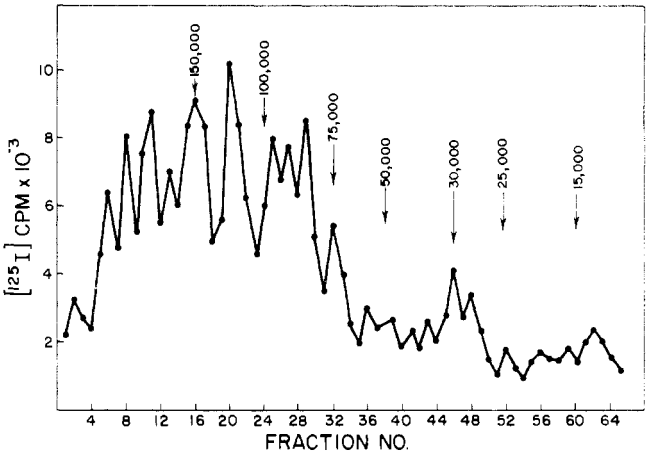

FIGURE 2: Distribution of ^{125}I in polyacrylamide gel of total rat embryo nonhistone chromosomal proteins. Gels were electrophoresed, serially sectioned, and assayed as described. Relative molecular weight determinations were calculated following similar electrophoresis of bovine serum albumin, trypsin, hemoglobin, ribonuclease, aldolase, and chymotrypsin calibration standards.

fied DNA preparations of all sources averaged between 30 and 60 × 10^6 as determined by 1.4% agarose slab-gel electrophoresis in the manner of Sugden et al. (1975) using several calibration standards. In each instance of affinity column preparation, greater than 90% of the DNA was adsorbed to the nonionic cellulose following the lyophilization and UV irradiation steps. Furthermore, nearly 90% of bound, ^3H-labeled DNA remained attached to each respective cellulose column following extensive usage and storage at 5 °C. In particular, BrdUrd-substituted DNA affinity columns were equally stable despite the incorporation of the photosensitizing analogue (Ehrlich and Riley, 1974). Since the total amount of substitution by bromouracil was slight (<7%) (Schwartz, 1976), significant molecular damage and disintegration were minimal, as determined by periodic assays for [^3H]bromouracil bound to cellulose. [^3H]BrdUrd-labeled DNA-cellulose affinity columns were also prepared with omission of the final UV irradiation step. Although the absolute efficiency of nucleic acid binding to cellulose was reduced nearly 50% in this instance, the subsequent elution profiles of adsorbed NHCP were relatively similar to columns prepared by irradiation. Since it was reported that rat liver nuclei contained nearly twice as much DNA as total acidic nuclear proteins in vivo (Teng et al., 1971), an equal mass of each component was reacted in vitro through affinity chromatography to assure maximal interaction. In most instances, nearly 300 000 cpm of ^{125}I-labeled NHCP (approximately 200 μg of protein) were applied to each DNA-cellulose column (approximately 200 μg of DNA).

Table II demonstrates the typical results obtained from recombination by affinity chromatography. Little radioactivity was retained by DNA-free cellulose; however, a significant amount of iodinated rat embryo NHCP was initially adsorbed to homologous, as well as heterologous, DNA-cellulose columns. Most importantly, a significant difference in relative strengths of binding to rat DNA was evident. Nearly two-thirds of the DNA-binding proteins was dissociated from the untreated rat DNA columns with low ionic strength (0.2 M NaCl) elutions, as compared to 48% from BrdUrd-substituted DNA. The remaining DNA-binding proteins were ultimately eluted from the columns with increasing NaCl concentrations. However, the NHCP-BrdUrd-treated DNA reconstituted complexes were sufficiently more stable such that correspondingly higher Na^+ concentrations were required for subsequent protein removal. In all instances, greater than 85% of adsorbed NHCP was eluted following the 0.6 M NaCl wash. The elution of ^{125}I from all DNA-cellulose affinity columns was essentially quantitative following the last 2.0 M NaCl wash, in that <2% of ^{125}I remained bound.

Although some rat embryo NHCP demonstrated a binding affinity for both homologous and nonhomologous DNA, Table III reveals a relative species specificity. Small aliquots of each DNA-cellulose column type were weighed, boiled, centrifuged, and analyzed for free DNA at A_{260} using appropriate standards. Although each affinity column contained comparable amounts of DNA, significantly different proportions of iodinated rat NHCP were bound to each type. The final NHCP/rat DNA ratios were approximately 0.10 regardless of rat DNA origin or presence of BrdUrd. On the other hand, the NHCP/salmon sperm DNA proportion of 0.07, and the NHCP/calf thymus DNA proportion of 0.06 reflected a partial species specificity of binding.

Polyacrylamide Gel Electrophoresis of NHCP from Affinity Chromatography. In order to isolate and further characterize the DNA-binding population of ^{125}I-labeled rat embryo NHCP, DNA-cellulose affinity columns were again utilized. Following NHCP application, unbound radioactivity was removed from each column type with several column volumes of 0.05 M NaCl buffer as before, and residual radioactivity was subsequently eluted with 3 bed volumes of 2.0 M NaCl buffer. The total DNA-binding proteins were then concentrated and characterized by analytical polyacrylamide gel electrophoresis. The DNA-binding NHCP eluted from untreated rat DNA-cellulose (Figure 3A) contained multiple bands of ^{125}I following separation and electrophoresis. The major peaks of radioactivity ranged in molecular weight from approximately 10 000 to 140 000 with 80% of the separated species in the range of 22 000–100 000. Nearly comparable profiles were observed when the NHCP eluted from BrdUrd-treated DNA columns were similarly separated by disc gel electrophoresis (Figure 3B). Again, the majority of the banded proteins ranged from 20 000 to 80 000 daltons. These dispersed, heterogeneous patterns were in contrast to the profile of rat embryo NHCP eluted from salmon sperm DNA-cellulose columns (Figure 3C). Although multiple bands were

TABLE II: DNA-Cellulose Affinity Chromatography with ^{125}I-Labeled Rat Embryo Nonhistone Chromosomal Proteins[a]

	Blank cellulose	Salmon sperm DNA-cellulose	Calf thymus DNA-cellulose	Rat embryo DNA-cellulose	Rat liver DNA-cellulose	BrdUrd-substituted rat embryo DNA-cellulose
% ^{125}I-labeled NHCP bound to DNA-cellulose	2	19	15	15	14	19
% of bound ^{125}I-labeled NHP eluted by:						
0.2 M NaCl		58	60	62	64	48
0.6 M NaCl		28	27	25	25	40
1.0 M NaCl		6	7	6	4	5
2.0 M NaCl		6	5	6	5	5

[a] DNA-cellulose affinity columns were prepared according to the text. Approximately 200 μg of NHCP in 0.05 M NaCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA was applied to each DNA-cellulose column (previously equilibrated in the same buffer) at 5 °C. Following removal of the unbound nonhistone proteins, DNA-binding NHCP were eluted with several bed volumes of same buffer containing the increasing Na^+ concentrations shown.

TABLE III: Correlation of DNA-Binding, ^{125}I-Labeled Nonhistone Chromosomal Proteins with Heterologous and Homologous DNA Following Affinity Chromatography.[a]

	Salmon sperm DNA-cellulose	Calf thymus DNA-cellulose	Rat embryo DNA-cellulose	Rat liver DNA-cellulose	BrdUrd-substituted rat embryo DNA-cellulose
Total bound ^{125}I-labeled NHP (μg)	23.10	16.54	22.61	20.19	21.86
Total DNA on column (μg)	303.94	275.68	205.54	194.13	210.19
Protein/DNA (w/w)	0.07	0.06	0.11	0.10	0.10

[a] Quantitation of protein was performed according to Lowry et al. (1951). DNA adsorbed to cellulose was determined as described in the text.

observed as before, the molecular weight distribution was somewhat narrowed (11 000 to 80 000), and greater than 80% of the ^{125}I-labeled rat NHCP averaged between 25 000 and 50 000 daltons. In order to determine the relative degree of species specificity of the rat embryo DNA-binding NHCP, tandem DNA-cellulose columns were constructed. A small volume of rat embryo DNA-cellulose was poured first into a 20-cm glass column. A nylon disk was placed over the packed matrix before a suspension of calf thymus DNA-cellulose (which contained an equal mass of bound DNA) was layered above. For this application, a nonsaturating amount of NHCP was introduced directly to the calf thymus DNA column in order to prevent "spill over" into the rat embryo DNA-cellulose below. Following adsorption and removal of unbound protein, the upper calf thymus DNA-cellulose portion of the tandem column was carefully removed with a Pasteur pipet. The resultant 2.0 M washes obtained from the remaining column contained the subfraction of DNA-binding rat NHCP which demonstrated species specificity in relation to calf thymus DNA. Following electrophoresis of this material (which accounted for nearly 25% of the total DNA-binding population), a complex pattern of proteins was again resolved (Figure 3D). As in Figure 3C, the subfractionated rat-specific NHCP profiles included a broad range of high- to low-molecular-weight species. However, the major peak which accounted for nearly 80% of the radioactivity ranged in molecular weight from 30 000 to 70 000. It therefore appeared that a subgroup of rat embryo NHCP was isolated with a more finite range of molecular weights, and with an ability to distinguish homologous from heterologous nucleic acid nucleotide sequences.

Reconstitution of ^{3}H-Labeled DNA and ^{125}I-Labeled NHCP. In order to confirm and corroborate the data derived from affinity chromatography, a second method for the isolation of DNA-binding nuclear proteins was sought. The reconstitution system of Bekhor et al. (1969) and Teng et al. (1971) was utilized for this purpose. [^{3}H]Thymidine- and [^{3}H]BrdUrd-labeled rat embryo DNA samples were combined in solution with an excess of ^{125}I-labeled NHCP. The components were allowed to renature by stepwise dialysis against high to low ionic strength buffers. The reconstituted complexes were isolated by sedimentation through 5–25% sucrose velocity gradients (Teng et al., 1971). Purified, iodinated NHCP failed to enter the gradients to any significant extent when centrifuged in the absence of DNA (Figure 4C). On the other hand, treated and untreated rat embryo DNA banded similarly when sedimented in the absence of protein (Figure 4A,B). The low level of bromouracil incorporation minimized any significant density shift detectable by velocity centrifugation. Whenever nucleic acid–protein mixtures were sedimented through the gradients, comparable DNA-banding profiles were once again observed following ^{3}H assays (Figure 5A,B). However, in both these instances, a coincidental peak of ^{125}I was consistently detected within the gradient fraction containing the DNA peak. Isolation and subsequent analysis of the renatured complexes from both DNA types yielded DNA/protein ratios of approximately 10:1. These values were in agreement with the quantitative data obtained from affinity chromatography (Table III). Furthermore, iodinated immunoglobulin and ribonuclease failed to cosediment with radiolabeled DNA of either type when reconstituted and centrifuged in the same manner (data not shown). Accordingly, renatured rat NHCP–DNA complexes failed to cosediment when the ionic

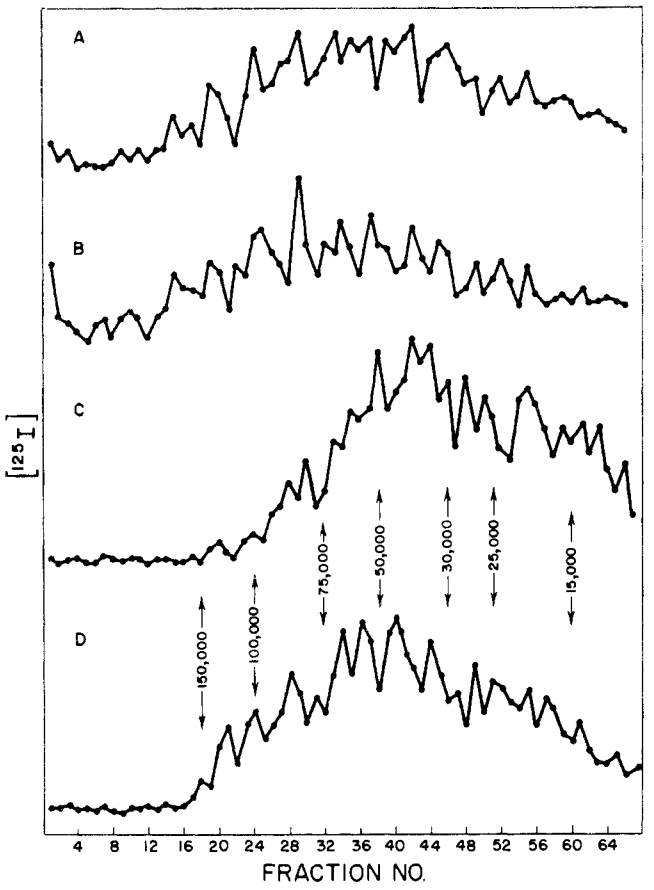

FIGURE 3: Relative distribution of ^{125}I in polyacrylamide gel electrophoresis of DNA-binding rat embryo nonhistone chromosomal proteins. Radiolabeled NHCP were removed from DNA-cellulose affinity chromatography and prepared for polyacrylamide gel electrophoresis as described. The ordinate represents cpm of ^{125}I within each gel slice fraction and ranges from 0 to 5000 cpm. (A) NHCP eluted from unlabeled rat embryo cell DNA-cellulose; (B) NHCP eluted from BrdUrd-substituted rat embryo cell DNA-cellulose; (C) NHCP eluted from calf thymus DNA-cellulose; (D) rat-specific NHCP eluted from tandem rat embryo/calf thymus DNA affinity column as described under Results.

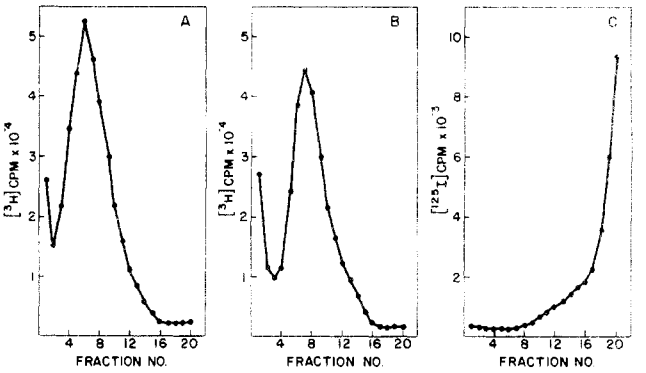

FIGURE 4: Distribution of radioactivity in fractionated 5–25% sucrose velocity gradients. Conditions of ultracentrifugation are described under Materials and Methods. (A) [^{3}H]Thymidine-labeled rat embryo DNA; (B) [^{3}H]BrdUrd-labeled rat embryo DNA; (C) ^{125}I-labeled NHCP. Direction of sedimentation is from right to left.

TABLE IV: Thermal Stability (T_m) of Native DNA and DNA-NHCP Reconstituted Complexes.[a]

	T_m (°C) of rat embryo DNA	T_m (°C) of BrdUrd-treated rat embryo DNA
DNA alone	87.5	87.8
DNA + NHCP	87.4	90.8

[a] DNA and DNA-protein complexes were adsorbed to hydroxylapatite as described. Single-stranded forms were eluted with 0.18 M phosphate buffer as the column temperature was stepwise increased in 2 °C increments (Schwartz, 1976). The values are the average of two determinations, and reflect the temperature at which 50% of the radioactivity had been eluted from the column.

strength of the sucrose gradients was raised to 2.0 M, in that the bulk of ^{125}I remained at the top of the gradient (Figure 5C).

Thermal Stability of Reconstituted DNA-NHCP Complexes. The gradient-purified nucleic acid-protein complexes were prepared for hydroxylapatite column chromatography as described. The thermal stabilities of these samples were compared to each other as well as to the respective native DNA types. Table IV demonstrates that the presence of bromouracil in purified rat embryo DNA did not significantly alter the melting temperature (T_m) when compared to the unsubstituted counterpart. Moreover, the presence of NHCP did not affect the T_m of the [^{3}H]thymidine-labeled complex. On the other hand, the stability of the BrdUrd-treated, reconstituted sample was increased such that a greater amount of thermal energy was required to denature the duplex molecule. These findings are in agreement with those of affinity chromatography (Table II).

Amino Acid Analysis of DNA-Binding NHCP. In order to more accurately characterize the qualitative nature of the DNA-binding proteins, amino acid analyses were determined from NHCP obtained from untreated and BrdUrd-substituted rat embryo DNA-affinity columns (Table V). In this instance, however, only those proteins eluted by buffers within the physiological range (0.1–0.6 M NaCl) were concentrated and analyzed. The overall distribution of amino acid residues was comparable between both DNA column types. However, a few differences existed when this differential pattern was compared to that of total rat embryo NHCP (Table I). Although the overall distribution was again enriched in acidic amino acid residues, serine and glycine were significantly elevated in both DNA-binding types.

Discussion

The highly specific nature of transcriptional alterations brought about by BrdUrd substitution in many biological systems has been extensively examined (Rutter et al., 1973). In most of these instances, incorporation of the analogue into chromosomal DNA was required in order to exert a characteristic effect. The most commonly observed responses subsequent to bromouracil replacement have been suppression (Stellwagen and Tomkins, 1971) or activation (Teich et al., 1973) of specific gene functions. We previously reported the induction and synthesis of a latent type C RNA virus from normal rat embryo cells exposed to low doses (10^{-7} M) of BrdUrd (Schwartz et al., 1974). Quantitative analysis revealed that fewer than 7% replacement of thymine by bromouracil was sufficient to achieve maximal endogenous virus expression (Schwartz et al., 1975).

These findings may partially reflect the increased stability of the substituted DNA–nuclear protein complex as described by others (David et al., 1974; Lapeyre and Bekhor, 1974). A functional prokaryotic counterpart was previously reported by Lin and Riggs (1972) in which an increased affinity of binding resulted between the lac operator and lac repressor in *Escherichia coli* DNA following BrdUrd incorporation. These data are generally compatible with a hypothesis in which bromouracil substitution promotes localized changes in

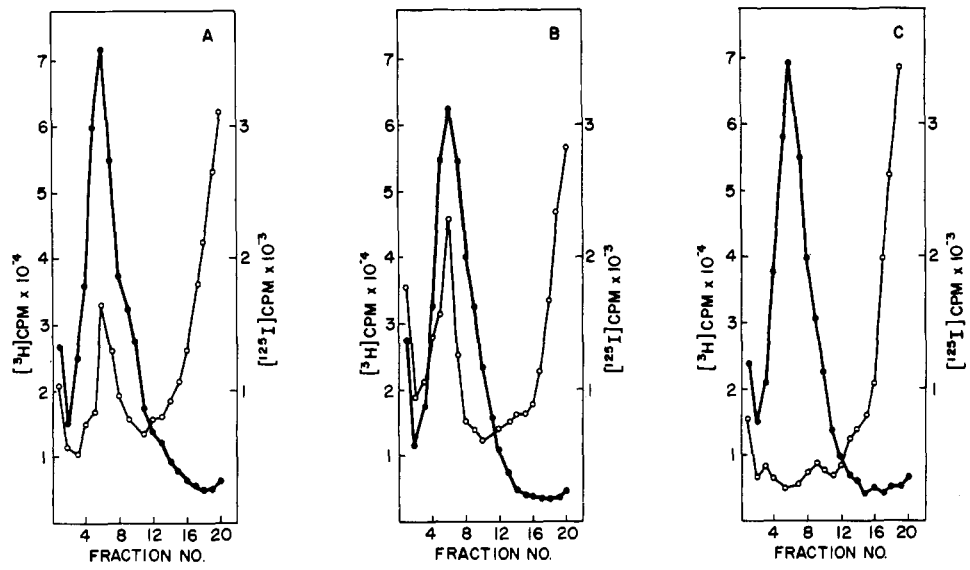

FIGURE 5: Distribution of radioactivity in 5-25% sucrose velocity gradients containing reconstituted DNA and NHCP complexes. (A) [^{3}H]Thymidine-labeled rat embryo DNA (●-●), ^{125}I-labeled NHCP (○-○); (B) [^{3}H]BrdUrd-labeled rat embryo DNA (●-●); ^{125}I-labeled NHCP (○-○); (C) reconstituted complex from A sedimented through a sucrose gradient made 2.0 M in NaCl as described under Results. [^{3}H]Thymidine-labeled DNA (●-●), ^{125}I-labeled NHCP (○-○).

chromatin such that native transcriptional regulation is impaired because of alterations to critical DNA–protein interactions, and/or failure of NHCP to recognize and bind to specific nucleic acid regions. The increase in electronegativity of the bromide constituent of BrdUrd as compared to the 5′-methyl group of thymidine may partially contribute to alterations in DNA–protein interactions.

A subfraction of rat embryo NHCP demonstrated an ability to bind in vitro to homologous and, to a slight extent, heterologous DNA. Nearly 20% of the NHCP mass was able to bind to DNA from any rat tissue source. The qualitative characterizations of DNA-binding NHCP were consistently similar, regardless of whether the recombinations took place on DNA adsorbed to cellulose or in solution during stepwise dialysis. Similar DNA-binding behavior has been described previously for NHCP extracted from many other tissues from several organisms (Stein et al., 1974). Despite the finding that similar types and amounts of rat embryo NHCP bound to BrdUrd-treated as well as to untreated rat DNA in vitro, the strength of binding was consistently and significantly greater with the bromouracil-substituted DNA.

Whereas the molecular weights ranged from 10 000 to 140 000 for total rat embryo NHCP, the predominant DNA-binding subfractions consisted of lower molecular weight species. van den Brock et al. (1973), Kleinsmith (1973), and others (Chiu et al., 1975; Wang et al., 1976) have reported similar molecular weight variations in DNA-binding proteins derived from several different rat tissues. Moreover, rat embryo DNA-binding proteins were quantitatively and qualitatively similar whether purified from affinity chromatography or stepwise gradient dialysis. These findings are consistent with the likelihood that the same, or highly similar, subfraction of nuclear proteins was consistently isolated, purified, and recombined with DNA. Sevall et al. (1975) and van den Brock et al. (1973) described rat NHCP families which exhibited species specificity when reacted with several heterologous DNA types. It was therefore not unexpected to discover comparable rat embryo NHCP subpopulations which shared these properties as well. Nearly 25% of total rat embryo DNA-binding NHCP was rat specific in comparison to DNA-cellulose columns of several heterologous DNAs. As

TABLE V: Amino Acid Composition of DNA-Binding NHCP.[a]

Amino acid	DNA-binding	BrdUrd-DNA-binding
Asp	8.5	8.4
Thr	4.4	4.6
Ser	13.1	18.7
Glu	11.8	12.3
Pro	6.0	4.6
Gly	21.0	20.7
Ala	8.3	8.9
Val	3.3	2.9
Ile	2.3	2.5
Leu	5.1	4.6
Tyr	1.9	1.2
ϕ Ala	2.3	2.1
Lys	3.6	4.6
His	5.1	2.1
Arg	2.9	1.7

[a] DNA-binding NHCP were eluted from affinity columns with buffers within a physiologic range (0.1–0.6 M NaCl) and prepared as before for amino acid analysis. Values given are in mol/100 mol of amino acids and are uncorrected for losses due to acid hydrolysis.

before, this particular population of NHCP, following separation by gel electrophoresis, was composed of many different protein bands of molecular weights ranging between 20 000 and 80 000. The biological significance of this protein subfraction remains to be clarified.

At the present time, NHCP preparations have been primarily utilized in functional experimental studies to measure relative rates of transcription from chromatin (Stein et al., 1974) or purified DNA templates (Teng et al., 1971) in vitro. At a mechanistic level, these studies have shown that the NHCP fraction substantially increased the rate of RNA synthesis. The presence of bromouracil in DNA with an overall increased potential for NHCP binding may result in a cellular inability to regulate certain genetic functions. Studies are currently in progress to examine this possibility. Although this simplified hypothesis is not new, the combined evidence of Nicolini et al. (1975), Lapeyre and Bekhor (1974), and the present work is altogether strong evidence that such a mech-

anism to account for BrdUrd-mediated effects at the transcriptional level is certainly tenable.

Recent evidence derived from nitrocellulose filter retention studies described increased and selective interaction between BrdUrd-substituted DNA and histone and non-histone chromosomal proteins (Lin et al., 1976; Gordon et al., 1976). Previously, Sevall et al. (1975) and Thomas and Patel (1976) reported NHCP with an affinity for rat DNA nucleotide sequences of different reiterative frequencies and base content. We reported the nonrandom localization of radiolabeled BrdUrd within moderately repeated DNA sequences of rat embryo cells induced to synthesize endogenous oncornavirus (Schwartz, 1976). Intensive investigations regarding the molecular interactions between these particular bromouracil-substituted regions of DNA and particular NHCP moieties are currently in progress in order to clarify the mechanism(s) of latent virus activation in vitro by BrdUrd.

Although data consistent with some of the general findings regarding molecular mechanisms for BrdUrd-mediated effects described here have been recently reported elsewhere, the rat embryo cell system contains an inherent advantage. Only minimal, nonrandom replacement of thymine by bromouracil (<7%) is sufficient to achieve a distinct biological effect (virogenesis). The commonly described extensive macromolecular modifications brought about by massive and random substitution by BrdUrd throughout the entire genetic material are thereby avoided. Therefore, selective "dissection" of the analogue-substituted DNA nucleotide sequences can be meaningfully correlated with the characterization of the responding NHCP moieties at enhanced levels of sensitivity, in contrast to isolated findings obtained from biological material representing nonphysiological states.

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