Carboxyl Methylation of Nonhistone Chromosomal Proteins†

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ABSTRACT: The in vitro methylation of nonhistone chromosomal proteins (NHCP) was investigated in nuclei isolated from the brain, liver, and thymus of 6–8-day-old rats. After the nuclei were incubated in the presence of 20 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 Ci/mmol), the NHCP were separated from histones on hydroxylapatite and fractionated further on sodium dodecyl sulfate–acrylamide slab gel electrophoresis. After the gels were dried, autoradiography was used to detect [^{3}H]methyl groups associated with these proteins. Four NHCP from the liver and thymus were methylated, while six methylated proteins were detected from the brain. None of the methylated proteins in these tissues corresponded with those from other organs, except for the component with a molecular weight of 66 000. It was evident that the methyl groups were esterified to the free carboxyl groups of NHCP since they are heat labile, yielding [^{3}H]-methanol. The carboxyl-methylated NHCP from these organs were tightly bound to chromatin. Nucleoplasm and loosely associated NHCP were essentially devoid of methylated proteins. The carboxyl methylation of NHCP was verified in vivo. Six-day-old rats were given L-[*methyl*-^{3}H]methionine (7 mCi/mmol) by intraperitoneal injection. The rats were killed at varying time periods and the NHCP isolated from gradient purified nuclei. Chromosomal nonhistone proteins, particularly from the liver, contained significant amounts of alkaline labile [^{3}H]methyl groups.

Eucaryotic chromatin is a complex of DNA, histone, nonhistone chromosomal proteins (NHCP),[1] and a small amount of RNA. The NHCP appear to have two roles. Certain of these proteins may serve in maintaining the structure of chromatin while others may be involved in gene regulation. More than half the mass of the NHCP found in vertebrates is composed of 12–18 proteins (Elgin & Bonner, 1970). In rat liver, six proteins account for 38% of the mass of NHCP. Four of these proteins have been identified preliminarily as myosin, actin, tubulin, and tropomyosin (Douvas et al., 1975). Like histone H3 and H4, these proteins do not turn over in the absence of cell division (Stambolova & Angelova, 1979; Srebreva et al., 1979). They contribute to the conformation of chromatin (Tashiro & Kurokawa, 1975) in the absence of histones (Adolph et al., 1977; Paulson & Laemmli, 1977; Defer et al., 1978; Chan & Liew, 1979; Bakayev, 1978) and are present in nucleosomes (Liew & Chan, 1976; Gronow et al., 1979; Brasch & Setterfield, 1974). These observations suggest that at least some NHCP may be involved in maintaining the structure of chromatin.

A great deal of evidence has accumulated which suggest that NHCP are involved in the regulation of gene expression (Baserga, 1974; Elgin & Weintraub, 1975; Stein et al., 1974, 1978; Stein & Stein, 1976). Changes in the composition and metabolism of various molecular weight classes of NHCP accompany modification in gene expression associated with development (Johnson & Hnilica, 1971; Shelton & Neelin, 1971), in vivo differentiation (Platz et al., 1975; Vidali et al., 1973), stimulation of cell proliferation (Stein & Burtner, 1975), a response to steroid hormones (Jensen & DeSombre, 1972; Tsai et al., 1976), and viral infection and transformation (Cholon & Studzinski, 1974; Krause et al., 1975). Chromatin reconstitution studies suggest that a component(s) of the NHCP may be involved in the tissue-specific transcription of the globin gene (Barret et al., 1974; Chiu et al., 1975), the steroid hormone induced transcription of ovalbumin (Tsai et al., 1976), and the cell cycle stage specific transcription of the histone genes (Stein et al., 1975; Jansing et al., 1977). These observations, including the fact that a substantial part of NHCP turnover in differentiating cells (Stambolova & Angelova, 1979; Kahl et al., 1979; Guguen-Gillouzo et al., 1979) and that certain NHCP are tissue specific (Teng et al., 1971; Miyazaki et al., 1978), strongly suggest that NHCP are involved in the regulation of gene expression.

The specific manner by which regions of the genome are regulated has yet to be defined. These proteins may control transcription by mediating structural changes in chromatin (Bekhor, 1978; Bekhor & Samal, 1977; Stein et al., 1977) and/or by resulting in the stimulation (Bekhor & Samal, 1977; Crepin & Dastugue, 1979; Legraverend & Glazer, 1980) or the inhibition of RNA polymerase (Kostraba et al., 1977). Bekhor & Samal (1977) have isolated a specific group of NHCP which stimulate RNA synthesis by an overall effect on the conformation of DNA/histones. Stein et al. (1977) have proposed that the histone gene is "derepressed" rather than "activated" by a component of the S-phase NHCP which modifies the interaction of histones with DNA in a specific manner to render this gene transcribable. Partial displacement of histones from DNA may be required. This could be brought about by competition of NHCP with specific sites on the DNA. One stimulatory fraction has been purified and characterized as having a molecular weight of 66 000 and a pI = 8.2–9.0 (Legraverend & Glazer, 1980). Whether this class of proteins functions as a regulatory factor according to its specificity for the RNA polymerase and/or DNA is not known.

Conformational changes in the structure of NHCP could affect their interaction with chromatin, resulting in transcriptional changes. One type of protein modification that results in conformational changes in NHCP is phosphorylation. Nuclear phosphoproteins have been implicated in gene regulation. They are tissue specific (Platz et al., 1970), change throughout the cell cycle (Platz et al., 1973), and have a high rate of turnover (Krause et al., 1975). Changes occur in both protein species and degree of phosphorylation induced by a variety of gene-activating stimuli, such as hormones (Ahmed & Ishida, 1971; Jungman & Schwepe, 1972; Bottoms &

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[1] Abbreviations used: NHCP, nonhistone chromosomal proteins; PMSF, phenylmethanesulfonyl fluoride.

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Jungman, 1975). Differences in the phosphorylation of nuclear proteins also have been demonstrated during carcinogenesis (Chiu et al., 1979, 1980) and viral transformation (Krause et al., 1975). Nonhistone chromosomal proteins are also modified by other means, including acetylation (Suria & Liew, 1974), poly(ADP ribosylation) (Hayaishi & Ueda, 1977), thiol oxidation (Gronow & Thackrah, 1974), and methylation (Friedman et al., 1969; Wallwork et al., 1977, Suria & Liew, 1974). In this paper we provide evidence that NHCP are carboxyl methylated in a tissue-specific manner.

Materials and Methods

Chemicals. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet) was purchased from ICN Chemical and Radioisotopes Division, Irvine, CA, and was diluted with unlabeled *S*-adenosylmethionine (AdoMet) to a specific activity of 1.0 Ci/mmol. L-[*methyl*-^{3}H]Methionine was purchased from Schwarz/Mann. All other chemicals except AdoMet and *S*-adenosylhomocysteine (AdoHcy) were purchased from Mallinckrodt Chemical Works or Sigma Chemical Co. AdoHcy and AdoMet were prepared as described by Duerre (1962) and Schlenk & DePalma (1957), respectively.

Assay of Carboxyl Methyltransferase in Nuclei. Nuclei from the brain and liver were purified as described previously (Duerre & Gaitonde, 1971). Kidney nuclei were prepared in a manner similar to liver nuclei. The thymus nuclei were not purified on a sucrose gradient because they lyse under such conditions. Therefore, they were washed once in 0.32 M sucrose/1.0 mM $MgCl_2$. Nuclei (approximately 10 mg of DNA/mL) were incubated at 37 °C in 20 μM [*methyl*-^{3}H]AdoMet (1.0 Ci/mmol), 0.32 M sucrose, 1.0 mM $MgCl_2$, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), and 50 mM potassium phosphate buffer, pH 6.9. The reaction was stopped at the designated time with the addition of AdoHcy to a final concentration of 0.5 mM and rapid cooling (or freezing) in a dry ice–ethanol bath. The nuclei were lysed in 7 volumes of 10 mM potassium phosphate buffer, pH 6.0, and 1.0 mM PMSF (2 mL/mg of DNA), and the chromatin was separated from the nucleoplasm by centrifugation. The chromatin was washed with 0.34 M sodium citrate buffer, pH 6.3 (2 mL/mg of DNA), with intermittant stirring for 5 min. After centrifugation, the [^{3}H]methyl groups incorporated into NHCP were released by the addition of 0.5 mL of 0.1 M NaOH. [^{3}H]Methanol was extracted with 3 mL of toluene/isoamyl alcohol (3:2 v/v) and measured by subtracting the radioactivity left after evaporation of a 1-mL aliquot from the total radioactivity present in a similar 1-mL aliquot. After the NHCP had been purified and fractionated on hydroxylapatite the residual counts after hydrolysis and extraction were near background. Protein concentration was determined by using Lowry reagent with an autoanalyzer.

Assay of Methylesterase Associated with Chromatin. Liver nuclei were incubated in the presence of [*methyl*-^{3}H]AdoMet for 40 min as described above. The nuclei were lysed with 4.0 mL of 10 mM potassium phosphate buffer, pH 6.0. The chromatin was collected by centrifugation and washed 3 times with the same buffer. It was suspended in 50 mM sodium acetate buffer, pH 5.2, containing 1.0 mM PMSF at 0 °C. The reaction was initated by incubating the chromatin at 37 °C. At the specified times, the reaction was stopped by adding 1.0 mL of 10% Cl_3CCO_2H. The picomoles of [^{3}H]methyl remaining per milligram of NHCP were determined as described previously. Controls were treated exactly as described above, except the methylesterase was denatured with 10% Cl_3CCO_2H prior to suspension in sodium acetate buffer.

Isolation of NHCP from Chromatin on Hydroxylapatite. NHCP were separated from histones and DNA on hydroxylapatite as described by Blüthman et al. (1975). Briefly, the chromatin was dissolved in 0.5 M NaCl, 5.0 M urea, and 1.0 mM sodium phosphate, pH 6.8 (buffer a) to give a final concentration of 0.5 mg of DNA/mL. This mixture was added to hydroxylapatite equilibrated previously in the above buffer. One milliliter of packed resin absorbs 0.7 mg of DNA or 1.0 mg of NHCP. After 30 min at 4 °C, the suspension was poured into a column (ratio of length to diameter 8:1) which was run at 4 °C with flow rate of 2–3 ml h^{-1} cm^{-2}. The unabsorbed histones H2A and H2B were eluted with the same buffer. The first nonhistone fraction was eluted with 0.45 M NaCl, 5 M urea, and 0.05 M sodium phosphate, pH 6.8 (buffer b), and the column equilibrated with the initial buffer (0.5 M NaCl, 5 M urea, and 0.001 M sodium phosphate, pH 6.8) to reduce the phosphate concentration to the original level. The second histone fraction was eluted with 2 M NaCl, 5 M urea, and 0.001 M sodium phosphate, pH 6.8 (buffer c). The remaining NHCP were eluted with a linear gradient formed by allowing 200 mL of 0.35 M potassium phosphate buffer, pH 7.5, 5.0 M urea, and 2.0 M KCl, to flow into an equal volume of 1.0 mM potassium phosphate, pH 7.5, 5.0 M urea, and 2.0 M KCl (buffer d).

Acrylamide Gel Electrophoresis. Slab gel electrophoresis was performed on gels containing 10% acrylamide as described by Laemmli (1970). Electrophoresis was carried out at 10 °C with a current of 30 mA.

Autoradiography. Autoradiography was performed by the method of Bonner & Laskey (1974). After the gel was destained, the water was removed with 50 volumes of dimethyl sulfoxide (Me_2SO) with two changes. The gel was placed in a 22.2% (w/v) solution of 2,5-diphenyloxazole in Me_2SO. After 3 h the Me_2SO was exchanged for water. The gel was dried and exposed to XR-5 Kodak X-ray film at −82 °C for up to 35 days.

Gas Chromatography. Analysis of volatile ^{3}H products was carried out on a Perkin-Elmer 900 gas chromatograph containing a 30-cm column packed with Porapak resin, type QS, 80–100 mesh, equipped with a column splitter which allowed approximately 30% of the effluent to bypass the detector (Toews & Adler, 1979). This effluent was collected (0.5-min fractions) by bubbling it through 10 mL of scintillation counting fluid. The injector and detector manifolds were maintained at 250 °C while the sample was injected at a column temperature of 50 °C. After a 1-min postinjection period, the column temperature was increased to 80 °C at a rate of 32 °C/min. Methanol was eluted from the column shortly after the temperature passed 65 °C.

Results

Fractionation of Methylated NHCP on Hydroxylapatite. After the nuclei from rat livers were incubated with [*methyl*-^{3}H]AdoMet, the chromosomal proteins were fractionated on hydroxylapatite (Figure 1). The electrophoretic patterns of the proteins under the various peaks were identical with those obtained by Blüthman et al. (1975). Histones H2A and H2B are located under peak 1, while the other histones are found under peak 3. Autoradiography revealed that the radioactivity under peak 1 was not associated with proteins while that under peak 3 was incorporated into histones H3 and H4 (data not presented). Of the three NHCP peaks (2, 4, and 5), only those proteins under peak 2 contained significant amounts of [^{3}H]methyl groups (Figure 1). The methylated proteins located under the radioactive peak at the beginning of the gradient (peak 4) were found to identical with those

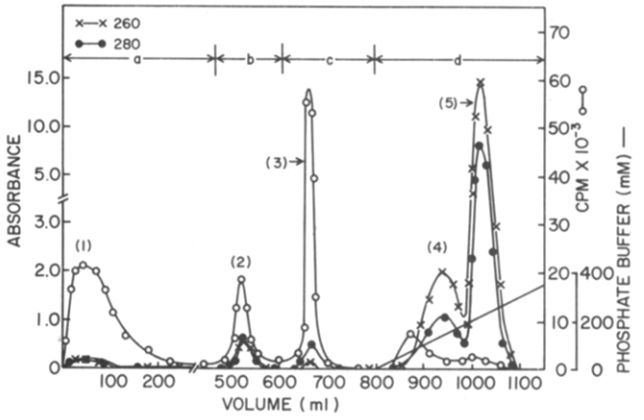

FIGURE 1: Fractionation of chromosomal proteins on hydroxylapatite. Gradient purified nuclei were prepared from 6–8-day-old rat livers as described under Materials and Methods. The nuclei (10 mg of DNA/mL) were incubated at 37 °C in 20 μM [*methyl*-^{3}H]AdoMet (1.0 Ci/mmol), 0.32 M sucrose, 1.0 mM $MgCl_2$, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), and 50 mM potassium phosphate buffer, pH 6.9. After 90 min, the nuclei were lysed with 10 mM potassium phosphate buffer, pH 6.0. The chromatin was separated from nucleoplasm by centrifugation, washed with 0.34 M sodium citrate, pH 6.3, and suspended in 0.5 M NaCl, 5.0 M urea, and 1.0 mM sodium phosphate buffer, pH 6.8. The NHCP were separated from histones on hydroxylapatite, as described under Materials and Methods.

under peak 2. Apparently all the methylated NHCP do not dissociate from the DNA in the presence of 0.45 M NaCl, 5.0 M urea, and 50 mM sodium phosphate buffer at pH 6.8 (buffer b).

Identification of the Type of Methylation of NHCP. Two milligrams of the NHCP under peak 2 (Figure 1) was incubated with 172 units of phospholipase C for 60 min at pH 7.0. The proteins were precipitated with 10% Cl_3CCO_2H and extracted with 5 mL of methanol/chloroform (2:1). Essentially all the radioactivity remained associated with the proteins, excluding the presence of methylated conjugated lipids.

The NHCP (2.3 mg) were hydrolyzed in 6 M HCl in vacuo and subjected to amino acid analysis (Wallwork et al., 1977). Contrary to a previous report (Friedman et al., 1969), these proteins were devoid of N-methylated amino acids, including lysine, arginine, and histidine. Furthermore, all the amino acids, including threonine and serine, were essentially devoid of radioactivity. Apparently the [^{3}H]methyl groups associated with these proteins were acid labile.

Two milligrams of the NHCP was subjected to hydrolysis in 0.5 mL of 0.1 M NaOH for 1 h at 37 °C. Microdistillation revealed that the radioactive product derived by alkaline hydrolysis was volatile at 65 °C. The boiling point of methanol is 64.5 °C. Recovery of the volatile radioactive product was achieved by microdistillation, extraction with 0.2 mL of toluene/isoamyl alcohol (3:2), and microdiffusion using a standard Conway cell (Kleene et al., 1977). Samples (1–5 μL) of the contents of the outer well of the Conway cell and the toluene/isoamyl alcohol extract were analyzed with the aid of a Perkin-Elmer gas chromatograph as described under Materials and Methods. The only radioactive compound detected cochromatographed with authentic [^{14}C]methanol. The contents of the Conway cell (1.5 ml) were mixed with 100 mg of nonradioactive methanol to verify the identity of the volatile product. This mixture was reacted with 3,5-dinitrobenzoyl chloride as outlined by Neish (1957) and the resultant product crystallized twice from a pyridine/water mixture. Eighty percent of the radioactivity was recovered in the resultant methyl 3,5-dinitrobenzoate (mp 107 °C). Protein methyl esters are the only known methyl derivatives of amino acids which are known to be unstable under acid and alkaline conditions (Kim et al., 1980; Kleene et al., 1977; Diliberto & Axelrod, 1974). The [^{3}H]methanol was most likely derived from the methyl esters of glutamic acid and/or aspartic acid.

Tissue Specificity of Carboxyl-Methylated NHCP. The hydroxylapatite fraction (peak 2) of the NHCP from brain, liver, and thymus showed distintive difference in electrophoretic components (Figure 2A). In the liver four of these components were methylated. The protein with a molecular weight of 66 000 contained by far the most radioactivity (Figure 2B). Brain chromatin exhibited 6-methylated components, the most intense have molecular weights of 35 000 and 66 000. Thymus

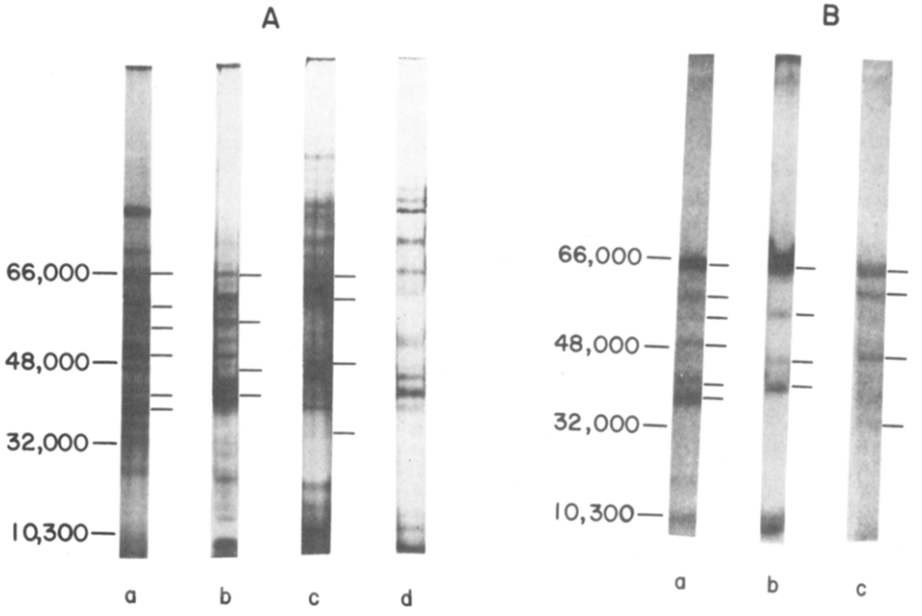

FIGURE 2: Electrophoresis (A) and autoradiography (B) of nonhistone chromosomal proteins. The nuclei were isolated from the brain (a), liver (b), and thymus (c) of 6–8-day-old rats and incubated with [*methyl*-^{3}H]AdoMet, and the NHCP were separated from histones and DNA as described under Materials and Methods. Approximately 50 μg of NHCP from peak 2 was subjected to slab gel electrophoresis in sodium dodecyl sulfate containing 10% acrylamide. The gels were stained with Coomassie Brilliant Blue to render the proteins visible, and autoradiography was performed as described under Materials and Methods. The letter d denotes liver nucleoplasmic proteins. The lines adjacent to the gels in (A) denote those proteins that are carboxyl methylated as observed in (B).

Table I: Incorporation of [^{3}H]Methyl Groups into Nuclear Fractions[a]

fraction	protein (mg)	total [^{3}H]methyl incorpd (cpm/mg)	[^{3}H]methanol cpm/mg	[^{3}H]methanol pmol/mg
nucleoplasm	7.8	23 000	300	0.5
loosely bound NHCP	4.2	17 500	630	1.1
chromatin				
histones H2A and H2B	1.8	19 100	780	1.4
NHCP (peak 2)	0.9	65 000	50 100	87.6
histones H1, H3, and H4	0.6	17 000	110	0.2
NHCP (peak 4)	0.5	8 000	3 000	5.2

[a] Liver nuclei were prepared and incubated with [*methyl*-^{3}H]-AdoMet, and the nucleoplasm was separated from chromatin, as described under Materials and Methods. Nucleoplasm was subjected to centrifugation for 1 h at 78000*g* to pellet the ribosomes. Chromatin was washed with 7.0 mL of 0.34 M sodium citrate, with intermittent stirring for 5 min, and the supernatant fluid was recovered by centrifugation. The chromatin was fractionated on hydroxylapatite as outlined under Figure 2. Proteins were analyzed for total radioactivity and [^{3}H]methanol as described under Materials and Methods.

chromatin did not contain any highly methylated components. Except for the component with a molecular weight of 66 000, none of the methylated proteins in these tissues correspond with those from other organs.

Properties of Carboxyl-Methylated NHCP. The carboxyl-methylated proteins are tightly bound to chromatin (Table I). Nucleoplasm is essentially devoid of methylated proteins, as are those proteins which are readily dissociated from chromatin with 0.34 M sodium citrate. However, the liver nucleoplasm contains proteins with molecular weights similar to that of methylated NHCP (Figure 2).

Detection of NHCP Methylesterase(s) Activity. The time course of incorporation of [^{3}H]methyl groups into NHCP in intact liver nucei is presented in Figure 3A. The methylation of these proteins proceeded at a linear rate for 10 min, reaching saturation after 40 min. If the incubation was continued after the methyltransferase reaction was blocked with AdoHcy, a gradual decrease in the amount of protein methyl esters was observed. This decrease could have resulted from either chemical hydrolysis or the presence of a methylesterase. Since all rat tissues contain methylesterases (Gagnon, 1979) and the methyl ester bonds are stable at a pH value below 8.5, the decrease was due probably to a methylesterase. When chromatin was washed extensively with 10 mM phosphate buffer, pH 6.0, methylesterase activity was still observed (Figure 3B). The further addition of nucleoplasm had no effect on this reaction, indicating that this enzyme was bound to chromatin. Whether or not this methylesterase is specific for NHCP is not known.

In Vivo Methylation of NHCP. The cytoplasmic fraction was rich in protein carboxyl methyltransferase activity (data not presented); however, this fraction and the microsomes were devoid of detectable radioactive carboxyl methyl groups, whereas trace amounts were detectable in the mitochondrial fraction (Table II). In agreement with the in vitro data was the finding that the firmly bound NHCP (peak 2) were rich in carboxyl-methylated proteins. To date we do not have accurate turnover data; however, this fraction was found to be devoid of radioactive labile carboxyl methyl groups within 20-h postinjection, indicating a very rapid turnover rate.

Discussion

When nuclei from the brain, liver, or thymus were incubated with [*methyl*-^{3}H]AdoMet, significant amounts of [^{3}H]methyl

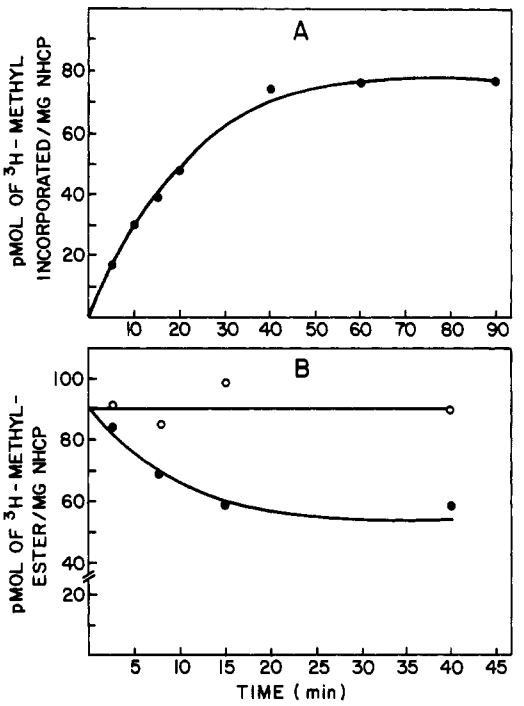

FIGURE 3: Incorporation and release of [^{3}H]methyl groups from NHCP. (A) Gradient purified liver nuclei were prepared and incubated with [*methyl*-^{3}H]AdoMet, and the reaction was stopped at the designated time periods with the addition of AdoHcy to a final concentration of 0.5 mM. Chromatin, prepared as described under Figure 1, was resuspended in 0.5 mL of 0.1 M NaOH for 60 min at 37 °C with intermittent mixing to release the radioactive methanol from the NHCP. The radioactive methanol released was extracted and measured as described under Materials and Methods. (B) After the nuclei were incubated with [*methyl*-^{3}H]AdoMet for 60 min, the chromatin was recovered as described above and suspended in 50 mM sodium acetate buffer (pH 5.2)/1.0 mM PMSF at 0 °C. The reaction was initiated by incubating the chromatin at 37 °C and stopped with the addition of 1.0 mL of 10% Cl_3CCO_2H. Controls (O) were treated exactly as described above, except the methylesterase was denatured with 10% Cl_3CCO_2H prior to suspension in the sodium acetate buffer.

Table II: In Vivo Incorporation of L-[*methyl*-^{3}H]Methionine into Various Fractions of the Liver[a]

fraction	pmol of [^{3}H]methyl/mg protein	pmol of [^{3}H]methyl/mg methanol
cytoplasmic	12.6	0.01
mitochondrial	30.4	0.17
microsomal	58.0	nil
nuclear		
nucleoplasmic	11.9	nil
loosely bound NHCP	15.8	0.28
firmly bound NHCP	47.1	1.74
histones	9.1	0.08

[a] Five 6-day-old rat pups were given [*methyl*-^{3}H]methionine (14 μCi/g of tissue) by intraperitoneal injection. The livers were removed 8 h later, rinsed with cold 0.32 M sucrose, and homogenized, and the various fractions were prepared by differential centrifugation. The nuclei were purified on sucrose gradients and lysed in 10 mM phosphate buffer, pH 6.0, containing 1.0 mM PMSF (nucleoplasmic fraction). The chromatin was washed with 0.34 M sodium citrate, pH 6.3, to remove loosely bound NHCP. The histones were extracted with 0.4 M HCl and the NHCP/DNA complex was fractionated on hydroxylapatite as described under Figure 1. Proteins were analyzed for total radioactivity and [^{3}H]methanol as outlined under Materials and Methods.

groups were incorporated into NHCP. The radioactive methyl groups associated with NHCP were relatively stable at pH values below 8.5. When the methylated NHCP were suspended in Tris–glycine buffer, pH 8.5, for 3 h at 10 °C, 90–95% of the radioactivity remained associated with the

proteins. However, 80–85% of the [^{3}H]methyl groups were released when the [*methyl*-^{3}H]NHCP were suspended in 0.1 M sodium borate, pH 11, at 37 °C for 1 h. Complete hydrolysis was achieved in 0.1 M NaOH after 1 h at 37 °C or in 6 M HCl after 18 h at 110 °C, resulting in the formation of methanol. From these data we concluded that the NHCP contained methyl esters of glutamate and/or aspartate. However, we have not determined which dicarboxylic amino acid was methylated. Protein methyl esters synthesized by protein methylase II are known to be extremely unstable and hydrolyze to methanol under mild alkaline or acidic conditions (Diliberto & Axelrod, 1974; Kim & Paik, 1970; Kim et al., 1980; Kleene et al., 1977).

In order to establish that the NHCP were not contaminated with trace quantities of highly methylated proteins from other sources, we examined the proteins from various cell fractions for alkaline labile methyl groups. Erythrocytes and white blood cells as well as cytosol, microsomes, mitochondria, nuclear membranes, and histones failed to yield significant amounts of [^{3}H]methanol upon alkaline hydrolysis. We therefore concluded that we were investigating the carboxyl methylation of NHCP.

Carboxyl-methylated NHCP appear to be tissue specific (Figure 2). Several NHCP were methylated in each of the three organs tested while only one of these proteins (M_r 66 000) was present in all three. The methylated proteins with molecular weights less than 66 000 did not appear to be the result of the hydrolysis of larger proteins. When PMSF, a proteolytic inhibitor, was added to the incubation mixture, no change in the molecular weights was observed.

When NHCP are methylated, the conformation as well as the location of the substrate and/or enzyme is extremely important. When liver nuclei were incubated with [*methyl*-^{3}H]AdoMet, the methylated proteins were observed to the associated with the DNA/histone complex (Table I). However, when nucleoplasm was incubated with [*methyl*-^{3}H]-AdoMet, many more proteins were methylated. This could have resulted from a change in the location of the enzyme and/or substrate or from conformational changes associated with the substrate.

Methylesterase activity was observed to be associated with liver chromatin. This enzyme was bound tightly to the DNA/histone/NHCP complex (Figure 3B), evident by the fact that the activity of the enzyme did not vary when chromatin was washed extensively with 10 mM phosphate buffer and the addition of nucleoplasm had no further effect. Methylesterase activity also was detected in the nuclei isolated from the thymus. Whether or not this chromatin-bound enzyme was specific for NHCP was not determined. Gagnon (1979) has reported previously the presence of methylesterase activity in various rat tissue homogenates, including brain and liver.

The carboxyl methylation of NHCP was verified in vivo (Table II). After an intraperitoneal injection of L-[*methyl*-^{3}H]methionine, [^{3}H]methyl groups were readily detectable in NHCP isolated from the liver. Alkaline labile methyl groups were also detectable in other organs, including brain, thymus, and kidney, although at a much lower level (data not presented). The different amounts of radioactivity incorporated into NHCP in the various organs could be ascribed to different turnover rates of the NHCP and/or the state of development of these organs, assuming that each organ contained a similar concentration of L-[*methyl*-^{3}H]methionine in the nucleoplasm.

It is possible that methylation of certain NHCP results in the binding of these proteins to the DNA/histone/NHCP complex. This is supported by several observations. Methylated NHCP are tightly bound to chromatin (Table I). Nucleoplasmic and loosely associated chromosomal proteins are essentially devoid of methyl groups. However, this fraction contains proteins with molecular weights similar to those proteins that are methylated (Figure 2). Carboxyl methylation of the acidic NHCP results in the neutralization of negative charges, allowing for the interaction of these proteins with negatively charged DNA. These observations give support to the hypothesis that certain newly synthesized NHCP, present in nucleoplasm, are methylated and as a result tightly bind to chromatin.

It is attractive to suppose that the methylation and demethylation of specific proteins could activate or inactivate genes by the binding or release of these proteins from chromatin. Gene regulatory proteins are expected to possess certain characteristics, including tissue specificity, a rapid turnover rate, and the ability to stimulate or repress RNA polymerase activity. The carboxyl methylated NHCP appear to be tissue specific (Figure 2) and turn over rapidly (data not presented). Legraverend & Glazer (1980) have purified a NHCP from rat liver that stimulates RNA synthesis. Interestingly, this stimulatory protein has a molecular weight of 66 000, identical with that of one of the methylated NHCP present in brain, liver, and thymus. The possible involvement of carboxyl methylation of specific NHCP in the regulation of RNA polymerase should be investigated.

Acknowledgments

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