Untucht-Grau, R., Schulz, G. E., & Schirmer, R. H. (1979) *FEBS Lett. 105*, 244.
Walsh, C., Fisher, J., Spencer, R., Graham, D. W., Ashton, W. T., Brown, J. E., Brown, R. D., & Rogers, E. F. (1978) *Biochemistry 17*, 1942.
Williams, C. H. (1976) *Enzymes (3rd Ed.) 13*, 89-173.
Williams, C. H., Arscott, L. D., & Schulz, G. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2199.
Wilson, G. S. (1978) *Methods Enzymol. 54*, 396.
Yokoe, I., & Bruice, T. C. (1975) *J. Am. Chem. Soc. 97*, 450.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468.

Protein Carboxyl Methylation-Demethylation System in Developing Rat Livers†

John A. Duerre* and Hazel A. Fetters
Department of Microbiology, School of Medicine, University of North Dakota, Grand Forks, North Dakota 58202
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ABSTRACT: Protein carboxyl methyltransferase and protein methylesterase activity was assayed in various cell fractions prepared from rat livers. Significant amounts of protein carboxyl methyltransferase were detected in the cytosol and nucleoplasm. The cellular concentration of this enzyme paralleled development, activity being highest in the liver from young animals. If methylation was inhibited at any point during the reaction with *S*-adenosylhomocysteine, protein methylesterase activity was evident by a rapid decrease in carboxyl-methylated proteins. Protein methylesterase activity could be assessed by measuring the amount of [^{3}H]methanol present in reaction filtrates. After a 10-min lag, the rate of demethylation was equivalent to the rate of methylation. The turnover of methyl groups was primarily enzymatic, since little or no methanol was generated when adrenocorticotropic hormone was incubated with purified protein carboxyl methyltransferase. Assessment of protein methylesterase activity as a function of the amount of methanol in the reaction filtrates represents minimal values, since the resultant [^{3}H]methanol was metabolized rapidly via an alcohol dehydrogenase and/or oxidase. The rapid turnover of the protein methyl esters makes it difficult to assess the endogenous methyl acceptor proteins. Protein methyl esters were not detectable in any significant amounts in hepatic cell fractions in vivo; however, the nuclei contained measurable amounts of carboxyl-methylated proteins in vitro. These proteins are firmly bound to DNA but are not an integral part of the nucleosome. Analysis of the proteins, after fractionation on hydroxylapatite and sodium dodecyl sulfate-acrylamide gel electrophoresis, revealed that several non-histone chromosomal proteins were carboxyl methylated. The approximate molecular weights of these proteins were 172K, 106K, 98K, 81K, 66K, 62K, 52K, and 38K. Results of binding studies suggest that these proteins originate in the nucleoplasm, perhaps after synthesis in the cytoplasm.

Protein carboxyl methyltransferase activity has been found in various rat tissues (Axelrod & Daly, 1965; Liss & Edelstein, 1967). This enzyme catalyzes the transfer of methyl groups from *S*-adenosylmethionine to the free carboxyl group of glutamyl and/or aspartyl residues in various proteins to form methyl esters (Kim & Paik, 1970). The protein methyl esters have been reported to be quite labile, undergoing hydrolysis to form methanol under physiological conditions (Axelrod & Daly, 1965; Kim & Paik, 1970). However, the lability of protein methyl esters is most likely due to the presence of a protein methylesterase. Gagnon (1979) has reported the presence of a heat-labile methylesterase in various rat tissues which readily hydrolyzes protein methyl esters, yielding methanol.

In bacteria, the carboxyl methylation-demethylation system has been found to play a role in chemotaxis (Black et al., 1982). This system involves the transfer of methyl groups from *S*-adenosylmethionine to specific methyl acceptor proteins in the bacterial cell membrane. Subsequent hydrolysis of the glutamyl methyl esters, via a methylesterase, results in the regeneration of the methyl acceptor protein and methanol (Stock & Koshland, 1978).

The function of the protein carboxyl methyltransferase-protein methylesterase system has not been delineated in eucaryotes. Diliberto & Axelrod (1976) have suggested that such a system may play a role in neurosecretion since high levels of protein carboxyl methyltransferase activity were found throughout the brain. The high concentrations of the enzyme in nonneuronal tissues (Duerre et al., 1985; O'Dea et al., 1981) would suggest that the enzyme system functions in a more general role and may regulate one or more processes in eucaryotes. One proposed function closely related to bacterial chemotaxis is leukocyte migration. The movement of leukocytes in response to a chemoattractant was found to enhance protein carboxyl methylation (O'Dea et al., 1978; Venkatasubramanian et al., 1980). Furthermore, the chemotactic response could be blocked with the addition of methylation inhibitors (Cantoni et al., 1979). van Waarde (1982) and van Waarde & van Haastert (1984) have demonstrated an increase in carboxyl methylation during aggregation of *Dictyostelium discoideum* amoeba in the presence of cAMP. Most of the methyl groups were incorporated within 15 s and lost within 1 min. The transient nature of the methylation-demethylation of proteins in *D. discoideum* is comparable to that observed

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in bacterial chemotaxis. McFadden & Clarke (1982) suggested that the methylation–demethylation system functions as an age repair mechanism which couples the hydrolysis of D-aspartyl methyl esters with their restoration to the L configuration. Erythrocyte membranes have been reported to contain extracellular and intracellular D-aspartyl sites for methylation (O'Connor & Clarke, 1983; Green et al., 1983). Methylation also has been reported to increase with erythrocyte age (Barbar & Clarke, 1983). It has been suggested that the increase in methylation of membrane proteins may contribute to the rigid cytoskeleton of aged erythrocytes. The racemization repair system may restore plasticity to the erythrocyte membrane and influence the cells' life span.

Currently, some investigators are looking at methylation–demethylation as a biochemical signal. This system operates very rapidly, is transient in nature, and has the ability to occur independently of de novo protein synthesis (Duerre et al., 1985). In proteins from photoreceptors in bovine outer rod segments, methylation–demethylation has been suggested as the molecular mechanism by which these components create a light-triggered response (Swanson & Applebury, 1983). The marked decrease in carboxyl methyltransferase activity noted with age (Duerre et al., 1985; Gagnon et al., 1978) has led to the hypothesis that the methylation–demethylation system play a role in organ development. O'Dea et al. (1981) and Zukerman et al. (1982) have found that human monocytes display increases in both cellular carboxyl methyltransferase activity and endogenous methyl acceptor proteins concomitant with morphological changes which occur when this cell differentiates into a macrophage. In contrast, human pulmonary alveolar macrophages, fully differentiated cells, do not show any increase in enzyme activity while methyl acceptor proteins increased markedly.

Results of this study revealed that the liver from young rats was as good a source of protein carboxyl methyltransferase as most other organs. Since the cellular level of the enzyme paralleled development, it was thought pertinent to try and identify the methyl acceptor proteins in these cells. However, little or no protein methyl esters could be isolated from hepatocytes. Failure to detect significant quantities of carboxyl-methylated proteins may be the result of their rapid turnover. All fractions of the cell were found to catalyze the hydrolysis of protein methyl esters. Results from in vitro studies were somewhat more encouraging. The nuclei were found to contain methyl acceptor proteins with molecular weights of 172K, 106K, 98K, 81K, 66K, 62K, 52K, and 38K. Carboxyl-methylated proteins in the outer rod segment of 61K and 88K molecular weight have been reported by Swanson & Applebury (1983), while the major carboxyl-methylated proteins in neuronal tissue have been reported to be about 60K (Quick et al., 1981).

Experimental Procedures

Chemicals. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (15 Ci/mmol) and [^{14}C]methanol (10 μCi/mmol) were purchased from ICN Chemical and Radioisotope Division, Irvine, CA. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine was diluted to 1.0 Ci/mmol with *S*-adenosylmethionine (AdoMet)[1] prepared by the methods of Schlenk & DePalma (1957). All other chemicals were obtained from Sigma Chemical Co.

Preparation of Cellular Fractions. Long–Evans rats were killed by decapitation; the liver was removed and placed in 10 volumes of 0.32 M sucrose–1.0 mM $MgCl_2$. The tissues were homogenized with a Teflon–glass homogenizer and filtered through two to four layers of cheesecloth. Nuclei were sedimented at 800*g* for 10 min and further purified by density gradient centrifugation (Duerre & Gaitonde, 1971). The purified nuclei were lysed in 10 mM potassium phosphate buffer, pH 7.0. The chromatin was sedimented by centrifugation at 30000*g* for 20 min and the nucleoplasmic fraction dialyzed for 48 h against 20 mM phosphate buffer, pH 7.0, and 1.0 mM dithiothreitol with three changes.

The mitochondria were recovered from the supernatant fluid by differential centrifugation at 30000*g* for 20 min, while the microsomes were removed by centrifugation at 105000*g* for 1 h. These particulate fractions were washed twice in 0.32 M sucrose–1.0 mM $MgCl_2$ by repeated centrifugation. The cytoplasmic fraction was dialyzed as described above. All fractions were prepared and stored at −40 °C for several months without the loss of protein carboxyl methyltransferase activity.

Protein Carboxyl Methyltransferase Assay. Various cell fractions (1–2 mg of protein) were incubated with 60 mM sodium acetate buffer, pH 6.5, 10 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1.0 Ci/mmol), 1.0 mg of methyl acceptor protein, and water to a final volume of 0.4 mL. The reaction was initiated with the addition of [*methyl*-^{3}H]AdoMet and incubated at 37 °C for the designated time periods. When the reaction mixtures contained particulate fractions, the reaction was terminated with the addition of 1.5 mM AdoHcy.[2] After removal of the bound proteins by centrifugation at 30000*g* for 15 min, the soluble proteins were precipitated with the addition of 0.1 volume of 100% Cl_3CCOOH. Protein precipitate was removed by centrifugation at 30000*g* for 15 min and the reaction filtrate stored at 4 °C for the determination of [^{3}H]methanol produced during the course of the reaction. The protein precipitate was washed 3 times with 10% Cl_3CCOOH. The [^{3}H]methyl groups incorporated into the proteins were released upon hydrolysis in 0.1 M NaOH at 37 °C for 1 h (Kim & Paik, 1970). The [^{3}H]methanol liberated was extracted with 3 mL of toluene–isoamyl alcohol (3:2 v/v) and quantitated by subtracting the nonvolatile radioactivity remaining after evaporation of a 1.0-mL aliquot from the total amount of radioactivity in an equivalent aliquot.

Protein Methylesterase Assay. Protein methylesterase activity was assessed by measuring the amount of [^{3}H]-methanol present in the reaction filtrates (0.4 mL) after incubation of various cell fractions with [*methyl*-^{3}H]AdoMet in the presence or absence of ACTH. After removal of protein precipitates as outlined above, the residual AdoMet was precipitated from the filtrate by the addition of 0.5 mL of 20% phosphotungstic acid. Approximately 10 μmol of nonradioactive AdoMet was added as carrier. After the mixture stood overnight at 4 °C, the precipitate was removed by centrifugation at 30000*g* for 10 min. After the addition of 1.0 mL of carrier methanol, [^{3}H]methanol was recovered by distillation at 45 °C under vacuum (740 mmHg). A 0.2-mL aliquot of the methanol was suspended in 10 mL of Brays counting solution and radioactivity determined in a liquid scintillation spectrometer. [*methyl*-^{3}H]AdoMet was found to contain 5.0% volatile radioactive products as purchased. If

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; ACTH, adrenocorticotropic hormone; NHCP, non-histone chromosomal protein(s); Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] This concentration of AdoHcy (1.5 mM) was sufficient to inhibit methylation 97%.

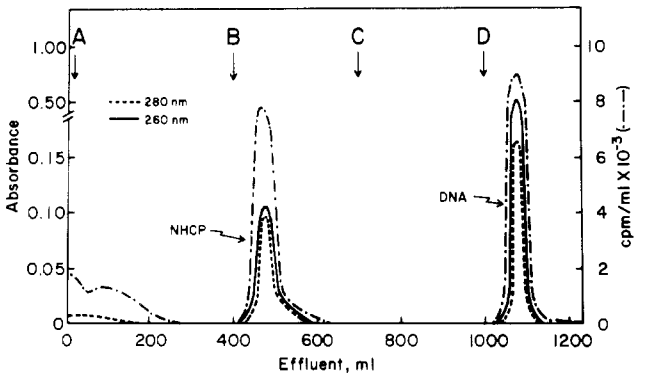

FIGURE 1: Fractionation of carboxyl methyl-^{3}H-non-histone chromosomal proteins on hydroxylapatite. Liver nuclei were incubated with *S*-adenosyl[*methyl*-^{3}H]methionine as described under Experimental Procedures. Chromatin, devoid of histones, was solubilized in 0.5 M NaCl, 5.0 M urea, and 50 mM sodium phosphate buffer, pH 6.8 (A). The chromatin was mixed with hydroxylapatite (1.2 mg of DNA/mg) and placed on a column (2.0 × 10 cm). The column was washed free of radioactivity with buffer A and the NHCP eluted with 0.45 M NaCl, 5.0 M urea, and 50 mM sodium phosphate buffer, pH 6.8 (B). The column was washed further with 2.0 M KCl, 5.0 M urea, and 1.0 mM sodium phosphate buffer, pH 6.8 (C). DNA was eluted with 2.0 M KCl, 5.0 M urea, and 0.35 M potassium phosphate buffer, pH 7.5 (D).

residual [*methyl*-^{3}H]AdoMet was not removed prior to distillation, up to 3.5% of the tritium was recovered as volatile radioactive products.

Isolation of NHCP from Chromatin with Hydroxylapatite. Non-histone chromosomal proteins were isolated as previously described (Quick et al., 1981). After nuclei were incubated with [*methyl*-^{3}H]AdoMet, the nuclei were lysed with 10 mM phosphate buffer, pH 6.0, and the chromatin was recovered by centrifugation. To remove loosely bound proteins, the chromatin was washed 3 times with the same buffer and twice with 0.3 M sodium citrate, pH 6.8. The histones were removed with 0.4 N HCl prior to dissolving the chromatin in 0.5 M NaCl–5.0 M urea–1.0 mM sodium phosphate, pH 6.8, to a final volume of 0.5 mg of DNA/mL. This mixture was added to hydroxylapatite resin which had been previously equilibrated in the same buffer. After gentle shaking for 30 min at 4 °C, the suspension was poured into a column (1.5 × 10.5 cm) which was run at a flow rate of 8–10 mL/h. The unadsorbed proteins and residual [*methyl*-^{3}H]AdoMet were eluted with the same buffer. The non-histone chromosomal proteins were eluted with 0.45 M NaCl–5.0 M urea–0.05 M sodium phosphate buffer, pH 6.8 (Figure 1). Protein was measured spectrophotometrically by the method of Warburg and Christian (Layne, 1957). A fraction of the protein was hydrolyzed in 0.5 mL of 0.1 M NaOH and assayed for [^{3}H]-methanol as described above.

SDS–Acrylamide Gel Electrophoresis. The molecular weights of carboxyl-methylated non-histone chromosomal proteins were determined by sodium dodecyl sulfate (SDS)–acrylamide gel electrophoresis as performed by the method of Laemmli (1970). The gel contained 10% acrylamide, 0.8% bis(acrylamide), 0.375 M Tris-HCl, pH 7.5, and 0.1% SDS. Electrophoresis was carried out at pH 6.5 at 10 °C with a current of 25 mA/gel. After being photographed, uniform gel slices (3 mm) were minced in 0.5 mL of 0.5 M NaOH. The vials were sealed and incubated at 37 °C for 2 h. After being cooled to 4 °C, the vials were uncapped, and the [^{3}H]methanol was extracted with toluene–isoamyl alcohol as described above. Some 80–85% of the alkaline labile counts present in the protein prior to electrophoresis were recoverable from the gels. If the gels were stored at −80 °C, there was a gradual loss in alkaline-labile methyl groups, particularly from the large molecular weight species.

Table I: Distribution of Protein Carboxyl Methyltransferase and Protein Methylesterase Activity in 21-Day-Old Rat Liver[a]

fraction	pmol of carboxyl [^{3}H]methyl incorpd/mg of protein		pmol of [^{3}H]methanol produced/mg of protein	
	endogenous	ACTH	endogenous	ACTH
cytoplasm	4 ± 1	34 ± 6	14 ± 2.5	28 ± 4
microsomes	2 ± 0.5	<1	1 ± 0.4	1 ± 0.5
mitochondria	<1	<1	0	1 ± 0.4
nuclei	8 ± 2	10 ± 2	5 ± 1.2	6 ± 1.0
nucleoplasm	4 ± 1	6 ± 1	0	1 ± 0.3
nuclei + cytosol	8 ± 1	11 ± 2	6 ± 1.2	17 ± 2

[a] The reaction mixture contained 0.32 M sucrose, 1.0 mM $MgCl_2$, 0.4 mM PMSF, 50 mM potassium phosphate buffer, pH 6.9, and 10 μM [*methyl*-^{3}H]AdoMet with or without 1.4 mg of ACTH and 2–3 mg of protein. After 30 min at 37 °C, the reaction was terminated with the addition of Cl_3CCOOH. Numbers are the mean ± SE of three determinations.

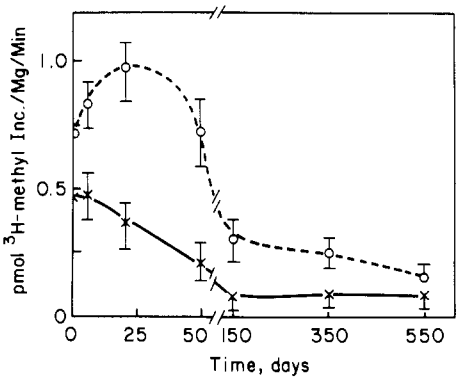

FIGURE 2: Level of protein carboxyl methyltransferase activity in rat liver during development and aging. Reaction mixtures contained 60 mM sodium acetate buffer, pH 6.5, 0.5 mg of ACTH, 10 μM [*methyl*-^{3}H]AdoMet (1.0 Ci/mmol), 0.3–0.6 mg of cytoplasmic (○) or nucleoplasmic proteins (×), and distilled water in final volume of 0.4 mL. The reaction was initiated with the addition of AdoMet and terminated with 10% Cl_3CCOOH after 6 min at 37 °C. Endogenous activity has been subtracted. Results are the mean ± SE of three determinations.

RESULTS

Cellular Distribution of Protein Carboxyl Methyltransferase. The cellular distribution of protein carboxyl methyltransferase was assayed by utilizing ACTH as the methyl acceptor and *S*-adenosyl-L-[*methyl*-^{3}H]methionine as the methyl donor. Of all the cell fractions from the liver, the most pronounced activity was observed in the cytoplasm prepared from 21-day-old animals (Table I). This activity was greater than or equal to that observed in most other tissues (Duerre et al., 1985). Rat liver has been reported to be one of the poorer sources of this enzyme (Gagnon, 1979; Kim & Paik, 1970). However, the age of the animal may be the major reason for this disparity in results. The cellular concentration of the enzyme was found to be directly dependent on the age of the animal. The most pronounced activity was observed in the cytoplasm during development with a 4-fold decrease in activity by day 150 (Figure 2). Significant enzyme activity also was detectable in the nucleoplasm during development, while the nucleoplasm was completely devoid of activity in the adult.

Endogenous methyltransferase activity was most pronounced in the nuclear fraction (Table I). In contrast, no significant increase in activity was observed when ACTH was incubated with nuclei, nor was this polypeptide readily methylated by

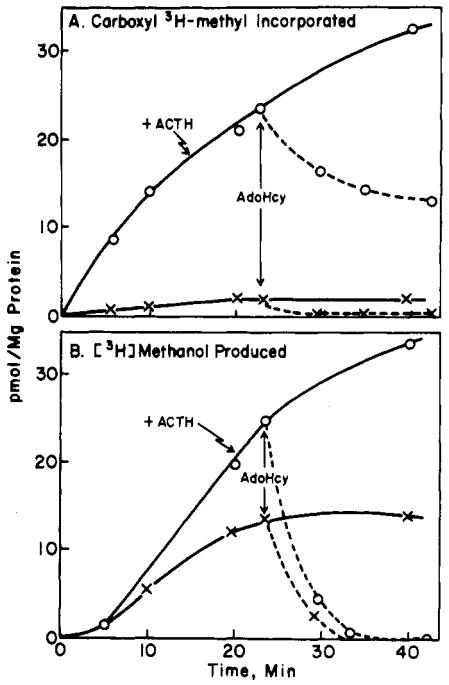

FIGURE 3: Methylation–demethylation of ACTH. (A) Rate of carboxyl methylation of ACTH (○) or endogenous (×) proteins and (B) rate of formation of [^{3}H]methanol. Reaction mixtures contained 10 μM [*methyl*-^{3}H]AdoMet and 60 mM sodium acetate buffer, pH 6.5, with or without 1.0 mg of ACTH and 1–2 mg of cytoplasmic protein prepared from 21-day-old rats. Methylation was inhibited by the addition of 1.5 mM AdoHcy as indicated by the dashed lines.

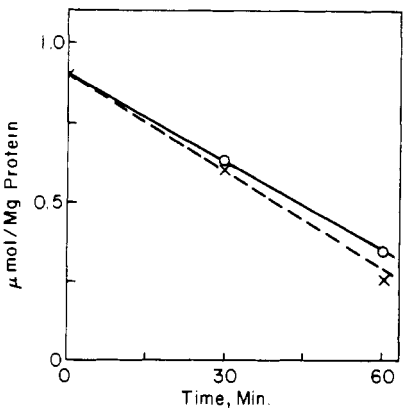

FIGURE 4: [^{14}C]Methanol metabolism by liver alcohol dehydrogenase and/or oxidases. The rate of methanol metabolism was determined by incubating 2–3 mg of liver cytosol (○) or nucleoplasma (×) with 60 mM sodium acetate buffer, pH 6.5, 16.7 mM [^{14}C]methanol (0.55 mCi/mmol), and distilled water to a final volume of 0.4 mL. The reaction was initiated by the addition of [^{14}C]methanol and terminated by the addition of 2.0 mL of 10% Cl_3CCOOH. Nonmetabolized [^{14}C]methanol was recovered from the reaction filtrates by distillation at 45 °C under a vacuum (740 mmHg). One milliliter of methanol was added as carrier.

nuclear soluble proteins (Table I). Similar results were obtained when nuclei were incubated with ACTH in the presence of cytoplasm. Apparently, the nuclei contain inhibitory substances or very active protein methylesterases.

Protein Methylesterase Activity. When ACTH was incubated with liver cytosol, the substrate appeared to be saturated within 40 min (Figure 3). At saturation, only 0.15 mmol of alkaline-labile [^{3}H]methyl groups had been incorporated per mole of ACTH. This represented less than 1 methyl residue per 6000 molecules of substrate. Failure to reach stoichiometric levels of methylation has been attributed to a number of factors such as product stability, enzyme stability, or the presence of protein methylesterases. After the resultant protein methyl esters had been purified by gel filtration and hydroxylapatite fractionation, they were found to be stable for several hours at pH 7.5 at 10 °C. Kim & Li (1979) also have found that the protein methyl esters were quite stable at neutrality.

Demethylation was apparent when the methyltransferase reaction was inhibited with AdoHcy (Figure 3A). Within 10 min of the addition of AdoHcy, the level of endogenous protein methyl esters returned to zero. There also was a decrease in methylated ACTH. The rate of demethylation was assayed by determining the amount of [^{3}H]methanol present in the reaction filtrates. After a 5–10-min lag period, the rate of formation of [^{3}H]methanol (1.2 pmol mg^{-1} min^{-1}) was equivalent to the initial rate of methylation (Figure 3B). The rate of methanol formation remained linear for up to 30 min after which time it appeared to reach saturation. Methanol appeared to be metabolized since the further addition of cytosol had no effect on the extent of alcohol production. This also was evident in the inhibition studies. When the methylation reaction was blocked with AdoHcy, the resultant [^{3}H]methanol disappeared within 10 min (Figure 3B).

Metabolism of methanol was assessed by measuring the disappearance of [^{14}C]methanol in the presence of various cell fractions. Methanol was metabolized at a rate of 8 nmol mg^{-1} min^{-1} in the presence of cytosol (Figure 4). Methanol also was metabolized by the nucleoplasmic fraction. This activity could account for the limited amount of alcohol detectable when ACTH was incubated with nucleoplasm (Table I). Consequently, the rapid disappearance of [^{3}H]methanol makes alcohol production an unreliable index of the extent of carboxyl methylation–demethylation. Subsequent attempts to inhibit methanol metabolism by the addition of ethanol were without success. The chelators EDTA and 1,10-phenanthroline have been found to be excellent inhibitors of alcohol dehydrogenase (Webb, 1963); however, attempts to selectively inhibit methanol metabolism with these chelators were unsuccessful.

Characterization of Protein Methyl Esters. Of all the cell fractions tested, the most highly methylated endogenous proteins were located in the nuclei (Table I). These protein methyl esters were firmly bound to chromatin since they could not be dissociated with dilute buffers or 0.35 M sodium salts, nor were significant quantities of the protein methyl esters dissociated from chromatin with 0.4 N HCl. When chromatin was subjected to limited digestion by staphylococcal nuclease, maximum solubilization of the protein occurred after 15% digestion. However, the protein methyl esters remained bound to the chromosomal fragments. Fractionation of nucleosomes on a Sepharose 4B 200 column revealed that the carboxylmethylated proteins were not an integral part of the nucleosome (Figure 5). As eluted from this gel, some 20% of the [^{3}H]methyl groups bound to the non-histone chromosomal proteins were alkaline-labile. Consequently, the non-histone chromosomal proteins were purified by passage through hydroxylapatite (Figure 1). Some 80% of the [^{3}H]methyl groups associated with the non-histone chromosomal proteins under peak 2 were alkaline-labile while all the [^{3}H]methyl groups associated with peaks 1 and 3 were alkaline-stable. The proteins under peak 2 were concentrated on a Amicon UM-2 membrane filter and analyzed by SDS–acrylamide gel electrophoresis at pH 7.5. The gels were fixed in acetic acid, stained, photographed, and sliced into 3-mm sections. The slices were minced in 0.5 M NaOH and analyzed for alkaline-labile [^{3}H]methyl groups as outlined under Experimental Procedures. The regions containing significant quantities of alkaline-labile [^{3}H]methyl groups correspond to Coomassie

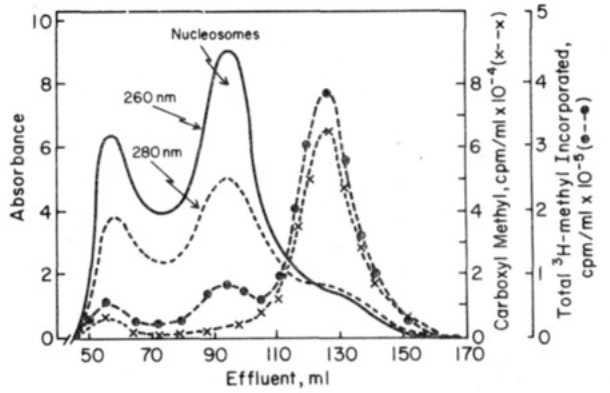

FIGURE 5: Fractionation of nucleosomes on Sepharose 4B-200. Nucleosomes and protein aggregates from a 15% nuclease digest were suspended in 0.6 M NaCl, 1.0 mM NaEDTA, 1.0 mM PMSF, and 5.0 mM potassium phosphate buffer, pH 7.0, and chromatographed on a Sepharose 4B-200 column (1.2 × 100 cm). Nucleosomes and soluble proteins were eluted with the same buffer. The proteins under the peaks were precipitated with 10% Cl_3CCOOH and analyzed for alkaline-labile [^{3}H]methyl groups.

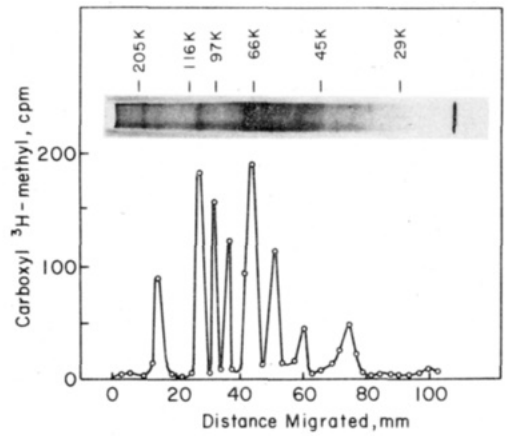

FIGURE 6: Characterization of carboxyl-methylated non-histone chromosomal proteins. Non-histone chromosomal proteins were extracted on hydroxylapatite as described under Figure 1. The proteins were separated according to molecular weight by SDS–acrylamide gel electrophoresis and visualized by staining with Coomassie blue. The bands containing carboxyl-methylated proteins were identified by slicing the gel into 3-mm segments and incubating the slices in 0.5 M NaOH. [^{3}H]Methanol was extracted with organic solvents and analyzed for alkaline-labile [^{3}H]methyl groups as described under Experimental Procedures.

blue stained bands at 172K, 106K, 98K, 81K, 66K, 62K, 52K, and 38K daltons (Figure 6). The protein methyl esters with molecular weights between 38K and 66K had been previously observed by autoradiography (Quick et al., 1981). Failure to observe the higher molecular weight species by autoradiography may be attributed to the drying procedure necessary for this technique and/or the length of time necessary to expose the photographic emulsion. If the acid-fixed gels were frozen, significant quantities of alkaline-labile methyl groups were lost within 2 weeks. This was most evident with the higher molecular weight species.

Origin of Methyl Acceptor Proteins. The cytosolic and nucleoplasmic proteins were incubated with or without the different particulate fractions to determine the possible origin of the methyl acceptor proteins. Activity was measured as a function of the amount of alkaline-labile methyl groups incorporated into protein as well as by measuring the amount of [^{3}H]methanol present in reaction filtrates. When nucleoplasm or chromatin was incubated alone, relatively few proteins were carboxyl methylated; however, when nucleoplasm was

Table II: Effect of Various Fractions on Carboxyl Methylation of NHCP[a]

	pmol of carboxyl [^{3}H]methyl incorpd/mg		[^{3}H]methanol produced	
	soluble protein	NHCP	cpm/mg[b]	pmol/mg
nucleoplasm	4.2		0	0
chromatin		3.0	0	0
nucleoplasm + chromatin	0.9	69.0	5280	8.6
nucleoplasm + chromatin + cytosol	2.1	55.0	7300	12.0
nucleoplasm + chromatin + microsomes	1.0	60.0	6330	10.2
cytosol	1.9		7200	11.7
cytosol + mitochondria	0.6	2.6	3720	6.0
cytosol + microsomes	2.5	4.3	10540	17.0

[a] Cytosol or nucleoplasm from 21-day-old rats was incubated with or without particulate fractions for 30 min at 37 °C. Soluble and particulate fractions were separated by differential centrifugation, and the NHCP were isolated on hydroxylapatite. Results are the average of two experiments. [b] Number of counts from a zero time control (4200 cpm) have been subtracted.

Table III: Binding of Carboxyl-Methylated Chromosomal Proteins to Chromatin[a]

	carboxyl methyl-^{3}H-NHCP	
system	cpm/mg of DNA[b]	% control
nuclei (1.0 mg of DNA)	1680 ± 160	
nuclei + cytosol (0.7 mg)	842 ± 120	50
nuclei + chromosomal proteins[c] (0.7 mg)	4350 ± 325	259
nuclei + cytosol + chromosomal proteins	4940 ± 410	294

[a] Rat liver nuclei from 22-day-old animals were incubated for 6 min at 37 °C with or without the cell fractions listed in the presence of [*methyl*-^{3}H]AdoMet. Numbers are the mean ± SE of two separate experiments. [b] About 0.1 mg of protein was obtained per milligram of DNA. [c] Chromosomal proteins were obtained by freeze–thawing liver chromatin and incubating for 1 h at 37 °C in the absence of AdoMet.

incubated with chromatin, a significant amount of carboxyl methyl groups were associated with the NHCP (Table II). The methyl acceptor proteins may be of nucleoplasmic origin since there was a 4-fold decrease in the amount of soluble carboxyl-methylated proteins when chromatin was incubated with nucleoplasm. The production of a significant amount of [^{3}H]methanol by the nucleoplasmic–chromatin system suggests that the NHCP were continuously being methylated and demethylated. When chromatin was incubated without AdoMet, some of the NHCP were released into the medium. After 1 h at 37 °C, about 0.1 mg of protein was solubilized per milligram of chromosomal DNA. If these proteins were incubated with nuclei, there was a significant increase in the amount of carboxyl-methylated proteins bound to DNA (Table III).

Although the cytosolic fraction was rich in protein carboxyl methyltransferase activity, it did not enhance the carboxyl methylation of NHCP (Table II). This may be due to the limited availability of methyl acceptor proteins or to elevated levels of protein methylesterase. When compared to nucleoplasm alone, a significant increase in the amount of [^{3}H]-methanol was observed whenever cytosol was incubated with endogenous proteins (Table II). The high affinity of the protein methylesterase for soluble endogenous carboxyl-methylated proteins could account for the limited extent to which these proteins accumulated when cytosol was added to the system. Attempts to inhibit protein methylesterase activity through the use of the protease inhibitors PMSF or diisopropyl

fluorophosphate were unsuccessful, while attempts to increase the level of methylated proteins via the addition of various methyl esters (phenylalanine methyl ester, glycine methyl ester, or lysine methyl ester) were only of marginal value.

Discussion

The incubation of rat liver nuclei with AdoMet resulted in the carboxyl methylation of several non-histone chromosomal proteins. Although liver cytosol was rich in protein carboxyl methyltransferase, it failed to enhance the methylation of these proteins. This may be the result of limited availability of protein methyl acceptor proteins or the presence of a protein methylesterase. Protein methylesterase activity was evident by the rapid disappearance of protein methyl esters when the methylation reaction was inhibitied with AdoHcy. Protein methylesterase activity also was manifest by the presence of [^{3}H]methanol in the reaction filtrates after cytosol or nucleoplasm had been incubated with AdoMet. Both the methylation and demethylation reactions were enhanced upon the addition of ACTH. Within 10 min, the rate of methanol formation was equivalent to the initial rate of methylation of ACTH. The rapid turnover of methyl groups was evident by the continued increase in methanol after endogenous substrates or ACTH appeared to be saturated.

Paik & Kim (1980) have suggested that the protein methyl esters are quite labile. However, we have found that the carboxyl-methylated non-histone chromosomal proteins are quite stable at neutrality. Furthermore, when ACTH was incubated with purified protein carboxyl methyltransferase (Kim et al., 1978), the reaction remained linear for up to 2 h without significant amounts of [^{3}H]methanol in the filtrate. This would indicate that hepatocytes contain large amounts of a protein methylesterase or that this enzyme has a relatively high affinity for the substrate.

The presence of a highly active protein methylesterase may contribute to the failure to accumulate protein methyl ester in vivo. When 10-day-old rats were given L-[*methyl*-^{3}H]-methionine, little or no alkaline-labile methyl groups were detected in any of the cell fractions from liver. Similar experiments have been conducted with a variety of cells in culture including hepatocytes, lymphocytes, K-B cells, and HL-60 cells. Significant amounts of radiolabeled methionine were incorporated by all of these cells. Some 15–20% of the [^{3}H]methyl groups were incorporated as protein methionine, while the remainder was utilized to methylate various macromolecules, particularly lipids, nucleic acids, and N-methylated proteins. Of the [^{3}H]methyl groups incorporated into protein, less than 1% were alkaline-labile.

McFadden & Clark (1982) have proposed that carboxyl methylation plays a role in the age-related D–L racemization repair system of aspartate residues in erythrocyte membranes as well as other proteins. O'Connor & Clark (1983) reported that erythrocyte membranes contain both intra- and extracellular sites for carboxyl methylation. When we incubated rat erythrocyte or hepatocyte membranes with protein carboxyl methyltransferase prepared from liver, we found no significant amount of protein methyl esters. Similar negative results were obtained with erythrocytes in vivo. Upon incubation of these cells with [*carboxyl*-^{14}C]methionine and [*methyl*-^{3}H]-methionine, the methylation index was 1. Furthermore, we were unable to isolate any radioactive phospholipids or protein methyl esters. All the radioactivity incorporated could be attributed to protein synthesis, most likely in reticulocytes. These data do not completely negate the possible function of carboxyl methylation in age racemization. However, failure to detect methyl esters in membranes and the marked decrease in the level of protein carboxyl methyltransferase in hepatocytes with age would not support this hypothesis. Billingsley et al. (1984) also have reported that they could find no evidence to support the hypothesis that only D-aspartate residues are responsible for carboxyl methylation.

Aswad (1984) recently reported that the carboxyl methylation of ACTH required the deamination of an asparagine residue at position 25. He further suggested that the chemical modification of Asn-Gly sequences during preparation of such proteins as ACTH, gelatin, or ribonuclease may account for their ability to accept methyl groups. The same line of reasoning may apply to calmodulin. Billingsley et al. (1984) found that less than 5% of calmodulin was carboxyl methylated. These investigators suggested that phosphodiesterases and/or protein kinases may indeed be the true substrates.

The elevated levels of protein carboxyl methyltransferase and methyl acceptor proteins in rapidly proliferating cells may implicate the system in growth and/or differentiation. Kloog et al. (1983) have found that protein carboxyl methylation parallels differentiation of neuroblastoma cells. Treatment of these cells with dimethyl sulfoxide, to induce morphological changes, resulted in an increase in protein carboxyl methyltransferase activity. The increase in enzyme activity closely parallels the development of the electrical excitability response. An elevation in protein carboxyl methyltransferase activity also has been observed in differentiating human monocytes and pulmonary alveolar macrophages in culture (Zukerman et al., 1982; O'Dea et al., 1981). The increase in enzyme activity during development and the identification of carboxyl-methylated proteins associated with chromatin may further implicate the system as having a role in the control of cellular differentiation at the genetic level. The methylation of a specific glutamyl or aspartyl residue would alter their charge and allow these proteins to bind to chromatin. The rapid reversal of this reaction via protein methylesterase would account for the presence of methyl acceptor proteins in the nucleoplasm in the absence of protein synthesis (Table III). The methylation–demethylation system may account for the rapid turnover of NHCP. It has been suggested that NHCP are involved in gene regulation since they turn over rapidly (GuGuen-Guillouzo et al., 1979; Krayse et al., 1975; Quick et al., 1981), vary throughout the cell cycle (Gerner & Humphrey, 1973), and have been correlated with changes in the metabolic state of the cell (Kostraba & Wang, 1973).

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References

Aswad, D. W. (1984) *J. Biol. Chem. 259*, 10714–10721.

Axelrod, J., & Daly, J. (1965) *Science (Washington, D.C.) 150*, 892–893.

Barbar, J. R., & Clarke, S. (1983) *J. Biol. Chem. 258*, 1189–1196.

Billingsley, M. L., Kuhn, D., Velletri, P. A., Kincaid, R., & Lovenberg, W. (1984) *J. Biol. Chem. 259*, 6630–6635.

Black, R. A., Hobson, A. C., & Adler, J. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 91–98, Macmillan, London.

Cantoni, G. L., Richards, H. H., & Chaing, P. K. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 155–164, Elsevier/North-Holland,

New York.
Diliberto, E. J., & Axelrod, J. (1976) *J. Neurochem. 26*, 1159-1165.
Duerre, J. A., & Gaitonde, M. K. (1971) *J. Neurochem. 18*, 1921-1929.
Duerre, J. A., Traynor, M. D., & Leatherbury, K. C. (1985) in *Progress in Nonhistone Chromosomal Proteins* (Beckhor, I., Ed.) Vol. 1, pp 103-116, CRC Press, Boca Raton, FL.
Gagnon, C. (1979) *Biochem. Biophys. Res. Commun. 88*, 847-853.
Gagnon, C. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R., & Creveling, C. R., Eds.) pp 55-64, Macmillan, London.
Gagnon, C., Viveros, J., Diliberto, E. J., & Axelrod, J. (1978) *J. Biol. Chem. 253*, 3778-3781.
Gerner, E. W., & Humphrey, R. M. (1973) *Biochim. Biophys. Acta 331*, 117-127.
Green, G., Sikka, S. C., & Kalra, V. J. (1983) *J. Biol. Chem. 258*, 12958-12966.
GuGuen-Guillouzo, C., Tichonicky, L., & Kruk, J. (1979) *Eur. J. Biochem. 95*, 235-238.
Kim, S., & Paik, W. K. (1970) *J. Biol. Chem. 245*, 1806-1813.
Kim, S., & Li, C. H. (1979) *Int. J. Pept. Protein Res. 13*, 282-286.
Kim, S., Nochumson, S., Chin, W., & Paik, W. K. (1978) *Biochemistry 17*, 415-422.
Kloog, Y., Axelrod, J., & Spector, I. (1983) *J. Neurochem. 40*, 522-529.
Kostraba, N. C., & Wang, T. Y. (1973) *Exp. Cell Res. 80*, 291-296.
Krayse, M. O., Kleinsmith, L. J., & Stein, G. S. (1975) *Life Sci. 16*, 1047-1058.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Layne, E. (1957) *Methods Enzymol. 3*, 447.
Liss, M., & Edelstein, L. M. (1967) *Biochem. Biophys. Res. Commun. 26*, 497-504.
McFadden, P., & Clarke, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2460-2464.
O'Connor, C., & Clarke, S. (1983) *J. Biol. Chem. 258*, 8485-8492.
O'Dea, R. F., Viveros, O. H., & Axelrod, J. (1978) *Nature (London) 272*, 462-464.
O'Dea, R. F., Viveros, O. H., & Diliberto, E. J. (1981) *Biochem. Pharmacol. 30*, 1163-1168.
Paik, W. K., & Kim, S. (1980) in *Protein Methylation* (Meister, A., Ed.) Vol. 1, pp 202-231, Wiley, New York.
Quick, D. P., Orchard, P. J., & Duerre, J. A. (1981) *Biochemistry 20*, 4724-4729.
Schlenk, F., & Depalma, R. F. (1957) *J. Biol. Chem. 229*, 1051-1057.
Stock, J. B., & Koshland, D. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3659-3663.
Swanson, R. J., & Applebury, M. L. (1983) *J. Biol. Chem. 258*, 10599-10605.
van Waarde, A. (1982) *FEBS Lett. 149*, 266-270.
van Waarde, A., & van Haastert, P. J. M. (1984) *J. Bacteriol. 157*, 368-3749.
Venkatasubramanian, K., Hirata, F., Gagnon, C., Corcoran, B. A., O'Dea, R. F., Axelrod, J., & Schiffman, E. (1980) *Mol. Immunol. 17*, 201-207.
Webb, J. L. (1963) in *Enzyme and Metabolic Inhibitors*, Vol. 1, p 84, Academic Press, New York.
Zukerman, S. H., O'Dea, R. F., Olson, J. M., & Douglas, S. D. (1982) *Mol. Immunol. 19*, 281-286.

Characterization of the Bovine Prothrombin Gene†

David M. Irwin,‡ Kevin G. Ahern,§ George D. Pearson,§ and Ross T. A. MacGillivray*,‡
Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5, and Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-6503
Received April 12, 1985

ABSTRACT: The bovine prothrombin gene was characterized by Southern blot analysis of bovine genomic DNA using bovine prothrombin cDNA fragments as hybridization probes. These analyses suggested that the bovine genome contains a single prothrombin gene that is at least 10 kilobase pairs (kbp) in size. To characterize the gene more thoroughly, two bovine genomic phage libraries were screened by using prothrombin cDNAs as hybridization probes. Heteroduplex analysis of the cloned genomic DNA and cDNA showed that the prothrombin gene is 14.9 kbp in size and contains at least 14 exons interrupted by 13 introns. The exons vary in size from 28 to 317 base pairs (bp), while the introns vary in size from <100 to 6940 bp. Regions of self-complementarity were observed within some of the introns, suggesting the presence of inverted repeat sequences. The bovine prothrombin gene shows similarities in structure to both the human prothrombin gene and the human factor IX gene.

The serine proteases are a family of structurally related proteins that are involved in a variety of essential physiological functions [for reviews, see Neurath & Walsh (1976) and Neurath (1984)]. These proteases are involved in both basic physiological processes, such as digestion and fertilization, and highly specialized physiological processes, such as the vertebrate immune response and blood coagulation systems. The serine proteases share regions of amino acid sequence identity particularly around the active site and the substrate binding sites (Hartley, 1970; Greer, 1981). This sequence identity is also found in prokaryotic serine proteases such as trypsin and proteases A and B from *Streptomyces griseus* (Olafson et al.,

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‡ The University of British Columbia.
§ Oregon State University.

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