Interaction of Histones with Estrogens. Covalent Adduct Formation with 16α-Hydroxyestrone†

Samuel C. Yu and Jack Fishman*
Laboratory of Biochemical Endocrinology, The Rockefeller University, New York, New York 10021
Received June 5, 1985

ABSTRACT: Disturbed estrogen metabolism leading to increased 16α-hydroxyestrone (16α-OHE) has been described in patients with systemic lupus erythematosus and mammary carcinoma. Previous studies showed the formation of covalent complexes between 16α-OHE and nonspecific cellular membrane proteins. The present study is concerned with the interaction of 16α-OHE and histones. Covalent adduct formation between 16α-OHE and individual histones was maximal with H1 histone. Other endogenous estrogens such as estrone, estradiol, and estriol did not interact with histones and form covalent adducts, nor did they interfere with the interaction of 16α-OHE with these nuclear proteins. The evidence supports that the adduct formation between 16α-OHE and histones proceeds via a stabilized Schiff base and subsequent rearrangement. This adduct formation which may have in vivo analogues may represent a mechanism for cellular transformation by this estrogen metabolite.

The mammalian metabolism of the female sex hormone is distinguished by its initial oxidation to estrone (Fishman et al., 1960) followed by hydroxylations at positions C-2 and 16α (Fishman, 1963; Brown & Marrian, 1957) which are to a large extent mutually exclusive. The principal, if not sole, products of 16α-hydroxylation are 16α-hydroxyestrone (16α-OHE) and estriol. While the biological profile of estriol has been extensively studied (Clark et al., 1977) and its similarity to that of the parent hormone identified, the physiological and pharmacological properties of 16α-hydroxyestrone have not been investigated in any detail. The recent observation that the formation of 16α-hydroxyestrone is elevated in patients with systemic lupus erythematosus (Lahita et al., 1981) has stimulated interest in the biological properties of this ketolic metabolite of estradiol. In competition experiments with radiolabeled estradiol the ketol was found to have only a very modest affinity for the rat uterine estrogen receptor, but nevertheless, it behaves as a potent estrogen in the in vivo rat uterotropic assay (Fishman & Martucci, 1980) and in the stimulation of the estrogen-sensitive MCF-7 cell proliferation (Schneider et al., 1984). The substance exhibits virtually no affinity for the human sex hormone binding protein (Fishman & Martucci, 1980), ensuring that the material in circulation in the human is biologically available. The most distinctive feature of this natural estrogen, however, is its ability to form covalent adducts with primary amino groups by a nonenzymatic process. This complexing occurs via Schiff-base formation followed by Heyn's rearrangement. The generation of such adducts with albumin and with erythrocyte and lymphocyte membrane proteins has now been demonstrated in vitro and in vivo (Bucala et al., 1982, 1984), providing a possible rationale for an etiological role for this substance in an autoimmune disease. Recently, this laboratory has found that the enzymatic activity responsible for the formation of 16α-hydroxyestrone is also elevated in women with breast cancer (Schneider et al., 1982) and in mouse strains with a high incidence of spontaneous mammary tumor formation (Bradlow et al., 1983). These findings suggested the possibility that the covalent interaction of this metabolite with nuclear components in estrogen target tissues could participate in the oncogenic process, with tissue specificity being conferred by the estrogen receptor which effectively localizes the substance in estrogen target cell nuclei. Basic nuclear proteins such as histones are likely candidates among nuclear components to undergo Schiff-base formation with carbonyl functions, and we therefore sought information on the interactions of 16α-OHE with these macromolecules in vitro. The results obtained confirm the relatively facile adduct formation between 16α-OHE but not other estrogens with histones. An analogous in vivo reaction may be a factor in estrogen-mediated cell transformation.

Materials and Methods

Chemicals were obtained as follows: acrylamide, amido black, ammonium persulfate, Bio-Gel P-60, methyl green, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine, and urea (Bio-Rad, Richmond, CA); charcoal Norit A, hydrogen peroxide, and sodium azide (Fisher, Springfield, NJ); glycerol (Mallinckrodt, St. Louis, MO); dextran T 70 and Sephadex G-25 (Pharmacia, Piscataway, NJ); 2-mercaptoethanol and sodium cyanoborohydride (Sigma, St. Louis, MO); 16α-hydroxyestrone, 17β-estradiol, estriol, and estrone (Steraloids, Wilton, NH).

Thymic histones purchased from Sigma contained heterogeneous classes of histones. Separation of homogeneous histone classes was achieved by chromatography of the histone mixture on Bio-Gel P-60 as described by van der Westhuyzen et al. (1974).

[6,7-^{3}H]Estradiol (40–60 Ci/mmol), [6,7-^{3}H]estrone (44 Ci/mmol), and [2,4,6,7-^{3}H]estriol (90–115 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [6,7-^{3}H]-16α-Hydroxyestrone (40 Ci/mmol) was synthesized as described previously (Ikegawa & Fishman, 1982). The 16α-tritiated estrone and estradiol were prepared by catalytic tritiation of 16,17-estrone enol diacetate followed by removal of the 17α isotope (Fishman, 1966) and had a specific activity of 40 Ci/mmol. The radiochemical purity of the labeled steroids was periodically checked by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). Thin-layer chromatography was carried out on silica gel G in

†Supported by Grant HD19825 from the National Institute of Child Health and Human Development and Grant AM07313 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

0006-2960/85/0424-8017$01.50/0

a solvent system consisting of chloroform/ethanol, 96/4 (v/v). The resulting TLC plate was scanned by a Packard radiochromatogram scanner. HPLC was performed on a Radial-Pak C_{18} cartridge (10 μm, 0.8 × 10 cm; Waters Associates, Milford, MA) using an isocratic solvent system of acetonitrile/water, 35/65 (v/v). The HPLC was interfaced with a Packard Trace to permit the identification of labeled steroids upon elution.

Reaction of Histones with [^{3}H]Estrogens. A typical experiment was carried out as follows: [6,7-^{3}H]-16α-Hydroxyestrone (1 μCi/tube) was added to microfuge tubes, the solvent was evaporated under nitrogen, and the steroid was redissolved with 20 μL of ethanol. Reaction was initiated by the addition of 1 mg of purified histones in 180 μL of 0.1 M potassium phosphate, pH 6.0. Where reducing conditions were desired, 1 μmol of sodium cyanoborohydride was included as the reducing agent. The mixtures were incubated at 37 °C with gentle shaking and aliquots drawn at specified times for analysis of binding.

Other ^{3}H-labeled estrogens were processed exactly as described. For competition studies radiolabeled 16α-OHE was mixed with an excess of unlabeled estrogen (1 mM) and added to the incubation. For electrophoretic analysis of the reaction products, 70 μg of histone was reacted with 2 μCi of [6,7-^{3}H]-16α-hydroxyestrone in the buffer system described above for 24 h. The reaction products were analyzed directly by acid–urea–polyacrylamide gel electrophoresis.

Except where stated otherwise, covalent binding was determined by the ethanol disk assay as described by Katzenellenbogen et al. (1975). Samples of 50 μL or less were spotted onto 2.4-cm glass microfiber filters (GF/B, Whatman, Clifton, NJ). These were collected and washed twice in boiling 95% ethanol, then rinsed twice at room temperature with ether/95% ethanol, 1/1 (v/v), again rinsed twice in pure ether, and then dried. All wash and rinse solutions were used at 3 mL/disk. The filters were then kept in Hydrofluor scintillant (National Diagnostics) for 10 h and then counted.

The charcoal–dextran was also used to remove unbound steroid. The 5% w/v slurry of charcoal coated with 1% dextran was used as 1 part to 10 parts of sample solution. The solution was exposed to the charcoal–dextran at 4 °C for 15 min with vortexing every 5 min. The suspension was spun at 1500*g* for 10 min, and the resulting supernatant was subjected to radioactivity measurement.

Samples of the reactions were electrophoresed in slab gels containing 15% acrylamide, 0.9 M acetic acid, and 2.5 M urea as described by Panyim & Chalkley (1969). Methyl green was run in side lanes as a tracking marker. Following electrophoresis, gels were fixed and stained for 4 h at 50 °C with 0.1% amido black in 0.7% acetic acid/30% ethanol and were then sliced into 5-mm slices. Each slice was treated with 1 mL of 30% hydrogen peroxide at 50 °C for 18 h and then counted.

Sephadex G-25 (20–50 μm) was swollen in 0.02 N HCl and then slurry packed into Econo-columns (0.7 × 20 cm, Bio-Rad) at 4 °C. After equilibration, a 0.4-mL aliquot of the reaction mixture was applied to the column and eluted with 0.02 N HCl in 0.25-mL fractions. The UV absorption of the fractions was measured in a Lambda 5 UV/vis spectrophotometer (Perkin-Elmer) and the radioactivity determined in a Packard 300 counter.

Acid hydrolysis of the 16α-OHE-modified histones was achieved in 6 N HCl at 110 °C under vacuum for 18 h.

In the experiments with [16α-^{3}H]estrone, two 50-μL aliquots were removed and diluted with 1 mL of buffer containing 0.5% charcoal. After centrifugation at 1500*g* for 10 min the supernatants were lyophilized to provide 0.5 mL of water which was counted in duplicate aliquots.

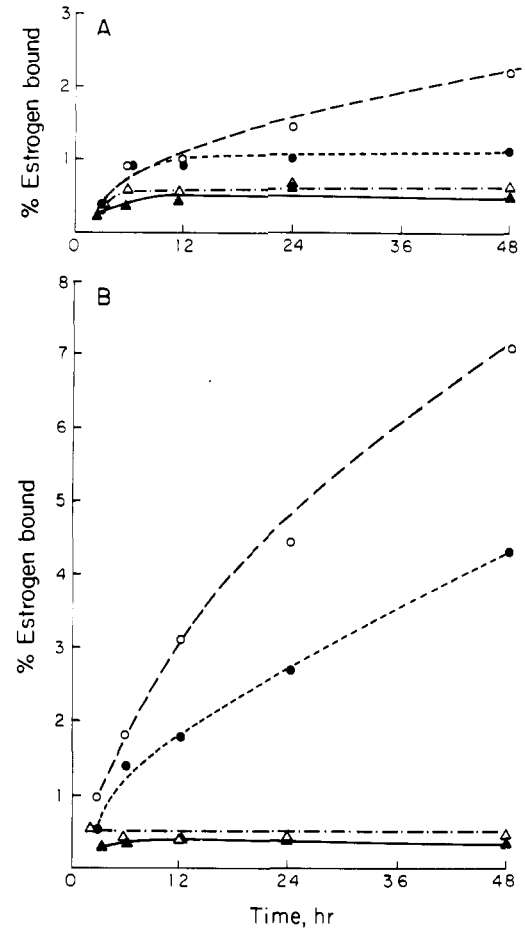

FIGURE 1: Time course of covalent labeling of H1 histone by [^{3}H]-estrogens in (A) the absence and (B) the presence of 5 mM $NaCNBH_3$. Thymic H1 histone (2.5 mg/mL) was incubated with [^{3}H]-estrogen (1 μCi) at 37 °C in 0.1 M potassium phosphate, pH 6.0/10% ethanol, and aliquots were assayed at specified times for adduct formation. The covalent adduct was assayed by ethanol extraction of aliquots spotted on filter disks. (○) 16α-Hydroxyestrone; (△) estriol; (●) estrone; (▲) estradiol.

RESULTS

Kinetics of Estrone–Histone Binding. The ability of several natural estrogens to form covalent adducts with histones is illustrated in Figure 1A. In order to avoid complication of reactions resulting from the addition of protease inhibitors, incubations were carried out at pH 6.0 with 0.1 M potassium phosphate/10% ethanol to minimize histone degradation. In the absence of a reducing agent, the incorporation of [^{3}H]-16α-hydroxyestrone into a histone adduct increased linearly with time reaching 2.5% of the substrate at 48 h. In contrast, less than 1% of [^{3}H]estrone was covalently bound to histone at 6 h, and this percentage did not increase further with time. Estradiol and estriol showed no detectable incorporation into histones during the entire 48 h of incubation.

In the presence of sodium cyanoborohydride, a reducing reagent that effectively reduces Schiff bases to secondary amines, the kinetics of the adduct formation would be expected to increase. Such results are shown in Figure 1B. The presence of the reducing agent resulted in a 3-fold increase in the incorporation of [^{3}H]-16α-hydroxyestrone into histone and caused also a progressive but lesser increase in [^{3}H]estrone adduct formation. The increase obtained in estrogen incor-

Table I: Efficiency and Selectivity of Histone Labeling with [6,7-^{3}H]-16α-Hydroxyestrone under Reducing and Nonreducing Conditions[a]

substrates	% bound	
	nonreducing	reducing
[^{3}H]-16α-OHE + histone	2.4	6.9
+ 16α-OHE	4.0	9.3
+ estrone	2.7	8.3
+ estradiol	2.5	6.4
+ estradiol	3.0	8.3

[a] Histone H2A/H2B (0.5 mM) was incubated with [^{3}H]-16α-OHE (1 μCi) at 37 °C in the absence or presence of unlabeled estrogen (1 mM) for 24 h. The extent of histone labeling was assayed by the charcoal method.

poration by sodium cyanoborohydride was consistently greater for 16α-hydroxyestrone than for estrone, indicating the greater presence of the Schiff base of 16α-hydroxyestrone compared to that of estrone and reflecting the stabilization of the former by hydrogen bonding with the hydroxyl group. The complex of [^{3}H]-16α-hydroxyestrone with histones involves a covalent bond since it does not dissociate in organic solvents. The reaction of the ketonic estrogens with histone is first order with respect to the steroid as demonstrated by the results of the dilution experiment (Table I). As expected from the mechanisms involved, the binding of 16α-hydroxyestrone to histone was not affected by the presence of excess estrone, estradiol, or estriol.

To examine possible contributions by non-histone contaminants to the adduct formation and to confirm the nature of the product, a preparation of [^{3}H]-16α-OHE-modified histone was subjected to electrophoresis in a polyacrylamide gel containing acetic acid–urea. Gel slices were counted to determine the distribution of bound [^{3}H]-16α-OHE. The two radioactive peaks detected were associated with the appropriate H2A/H2B monomer and dimer (Figure 2).

In the study to establish finally the covalent nature of the [^{3}H]-16α-OHE histone adduct, [^{3}H]-16α-OHE-modified histone obtained from the incubation mixture was subjected to chromatography on Sephadex G-25. The column fractions containing the histone complexed with [^{3}H]-16α-OHE were pooled and acid-hydrolyzed, and the resulting hydrolysate was then separated on the same chromatographic system (Figure 3). The radioactive peaks A–C corresponded to the intact histone adduct, the 16α-OHE–amino acid adduct, and the free 16α-OHE, respectively. The radioactivity derived from [^{3}H]-16α-OHE–histone adduct was entirely recovered in peak B, and none was found as free 16α-OHE after the acid hydrolysis. The amino acid contained in peak B was not determined, but it probably was lysine on the basis of the analogous albumin results.

Relative Histone Reactivity. By use of the acid–urea–polyacrylamide gel electrophoresis, the reactivity of five histone classes with 16α-OHE under nonreducing condition was compared. The results indicated the degree of labeling of the

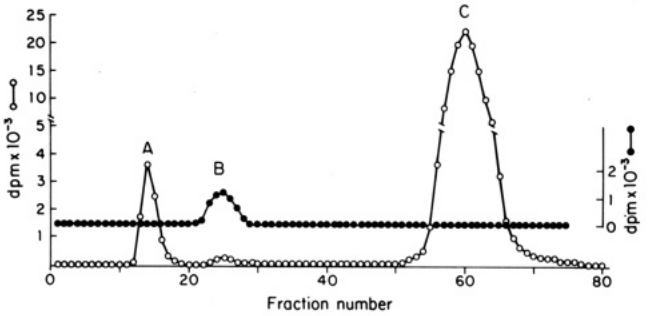

FIGURE 2: Electrophoretic analysis of histone H2A/H2B labeled with [^{3}H]-16α-hydroxyestrone on a polyacrylamide gel with acetic acid-urea. A portion of the reaction mixture was electrophoresed in polyacrylamide gel (15%) containing 0.9 M acetic acid and 2.5 M urea. The gel was stained with amido black and assayed for radioactivity.

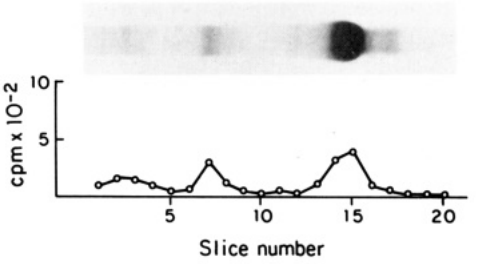

FIGURE 3: Chromatography of the [^{3}H]-16α-hydroxyestrone-modified histone H1 on Sephadex G-25 before (○) and after (●) acid hydrolysis. 16α-Hydroxyestrone-modified histone was isolated from the reaction mixture by the first chromatography on Sephadex G-25 (0.7 × 20 cm) and digested in 6 N HCl at 110 °C under vacuum for 18 h. The resulting hydrolysate was rechromatographed in the same system. Fraction volume was 0.25 mL. Peak A, intact histone-steroid adduct; peak B, amino acid–steroid adduct; peak C, free steroid.

histone with 16α-OHE proceeded in the decreasing order H1, H2B, H2A, H3, and H4. The relative amount of radioactivity associated with histone corresponded to the lysine content of the individual histone class.

The studies carried out with [16α-^{3}H]estrone and [16α-^{3}H]estradiol bear on the possible role of enolization in the interaction of ketonic steroids with histones. The very substantial transfer of ^{3}H from [16α-^{3}H]estrone but not [6,7-^{3}H]estrone or [16α-^{3}H]estradiol to water in the presence of histone with or without the concurrent presence of a reducing agent (Table II) provides convincing evidence of enolization of the 17-ketone. Since the amount of covalently bound radioactivity in the case of [16α-^{3}H]estrone is less than 50% of that with [6,7-^{3}H]estrone, it suggests that a disproportionate amount of the enolized product is involved in the Schiff-base formation which is trapped by reduction. This suggests that a similar enolization involvement exists in the case of 16α-hydroxyestrone. The small amounts of estradiol complexed with the histone may represent a product of photosensitized peroxidation which has recently been reported (Sedee et al.,

Table II: Structural Determinants on Estrone in the Formation of Covalent Adduct with Histone and the Concomitant Release of ^3H_2O from [^{3}H]Estrogen[a]

reagents	[6,7-^{3}H]estrone		[16α-^{3}H]estrone		[16α-^{3}H]estradiol	
	bound	released	bound	released	bound	released
control	0.08	0.33	0.03	0.88	0.03	0.18
histone	0.80	0.26	0.27	6.35	0.14	0.12
$NaCNBH_3$	0.08	0.28	0.04	0.80	0.04	0.13
histone + $NaCNBH_3$	2.53	0.23	1.21	4.76	0.21	0.12

[a] Histone (5 mg/mL) was incubated with [^{3}H]estrogen in 0.1 M potassium phosphate, pH 6.0/10% ethanol at 37 °C for 24 h. Two aliquots of the reaction mixture were assayed for the adduct formation by the ethanol disk method and two other aliquots assayed for the ^3H_2O release. Data are expressed as the percentages of total radioactivity bound to histone or released as ^3H_2O upon incubation.

1984) to contribute to covalent linkage formation.

Discussion

Our results demonstrated that histones are effective substrates for the formation of covalent adducts with the estradiol metabolite 16α-hydroxyestrone under in vitro conditions. The results also reveal that the ability of this ketolic metabolite to form these adducts derives from several factors. First, to undergo Heyn's rearrangement of the intermediate Schiff base, an adjacent hydroxyl group is required, a feature missing in ketonic estrogens such as estrone. Nonketonic estrogens such as estradiol and the 16α,17β-dihydroxy derivative estriol cannot form these adducts because they lack the essential ketone group for the formation of the initial Schiff base. The distinction is confirmed by our results in which neither estradiol nor estriol forms covalent complexes with histones even under reducing conditions. Estrone also does not react with histones to generate covalent adducts in the absence of reducing agents which serve to trap the intermediate Schiff base which otherwise is freely reversible to its constituents. A second factor assisting the adduct formation is the greater stability of the Schiff base derived from 16α-hydroxyestrone relative to that from estrone. This is evident from the almost 100% greater formation of 16α-hydroxyestrone adducts under reducing conditions relative to estrone. This implies that the free steroid–Schiff-base equilibrium is shifted more to the Schiff-base side in the case of the ketol, permitting more of it to be trapped under the reducing conditions. This equilibrium shift most probably reflects stabilization of the Schiff base by hydrogen bonding of the 16α-hydroxyl proton with the base nitrogen. This stabilization of the Schiff base also influences the amount of Heyn's rearrangement product which is formed in a specific time. Two other ring D ketols, 16-ketoestradiol and 16β-hydroxyestrone, which have the necessary structures for adduct formation via the above mechanism, are also metabolites of estradiol. Neither of these two ketols, however, is likely to form adducts by the above mechanism as readily as 16α-OHE. The thermodynamic stability of a ring D ketol containing a 16-ketone group is greater than that of the corresponding 17-keto isomer, and hence the Schiff base of 16-ketoestradiol is less likely to undergo the rearrangement that would require the generation of the thermodynamically unfavorable 17-keto structure (Fishman, 1960). On the other hand, 16β-hydroxyestrone undergoes the rearrangement to the isomeric 16-ketoestradiol so rapidly in the presence of base that this transformation would predominate in any equilibrium process involving Schiff-base formation. Therefore, the metabolite of estradiol that has the greatest potential for generating adducts with amino group by the Schiff base and Heyn's rearrangement process is 16α-OHE. Recently, it has been reported that the rearrangement of steroidal 16α-or 16β-hydroxy-17-ketones to the isomeric 16-keto-17β-ol proceeds via a mechanism (Numazawa et al., 1982) other than enolization mechanism. Heyn's rearrangement of the Schiff base may therefore proceed not by an enolization mechanism. Our results indicate that exposure of estrone to histones under the in vitro conditions employed does result in enolization as shown by the transfer of ^3H into water from [16α-^3H]estrone in contrast to the absence of such transfer in the case of [16α-^3H]estradiol, but whether this mechanism plays a role in Heyn's rearrangement is not known.

The clear potential that exists for adduct formation of 16α-OHE with basic nuclear proteins such as histones may have important consequences. Histones are integral structural components of chromatin and have a dynamic role in several aspects of gene function (Kornberg, 1977; Mathis et al., 1980). Binding of 16α-OHE to histone could interfere with accurate reading of the DNA template during transcription or replication. Additionally, 16α-OHE binding could stimulate or block an essential site for biochemical modification of nucleohistones during changes in gene function. The effects are likely to be long lasting because of the low turnover rate of core histones and possible lack of repair of chemical damage to polypeptides (Allfrey & Boffa, 1979).

The fact that the binding of 16α-OHE is most effective with lysine-rich proteins is intriguing. Antibodies to histones have been detected in patients with systemic lupus erythematosus, and lysine-rich histones H1 and H2B are the most prominent autoantigens (Harden & Thomas, 1983). H1 histone, the most lysine-rich molecule of this class of proteins and the one that exhibits the greatest adduct formation, occupies a unique position in chromatin (Littau et al., 1965; Sluyser & Snellen-Jurgens, 1970). Data obtained from in vivo (Cole et al., 1977) and in vitro (Weintraub & van Lente, 1974) experiments indicate that H1 histones may cross-link the DNA strands together, changing the distance between them. This is a basis for the assumption that it plays an active role in condensation and dispersion of chromatin in the process of cell division.

16α-OHE has been found to be an exceptionally potent uterotropic agent possibly exceeding even estradiol (Fishman & Martucci, 1980). The low affinity of 16α-OHE for sex hormone binding globulin permits ready access of this metabolite to the nucleus and its binding to chromatin. In view of the selective uptake of estrogen by its receptor in the target nucleus (Jensen et al., 1968), the effect of 16α-OHE on gene function may be exaggerated in estrogen-responsive organs and in humans. Under normal circumstances access of 16α-OHE to the target cell nucleus will be inhibited by the competition of estradiol and other estrogens for receptor's occupancy, but when the ratio of these estrogens to 16α-OHE is decreased, entry and binding of this ketol could occur. It is noteworthy that a positive correlation has been observed between estrogen 16α-hydroxylase activity, the enzyme responsible for 16α-OHE formation, and the incidence of breast tumors in women and experimental animals (Schneider et al., 1982; Bradlow et al., 1983).

Registry No. 16α-OHE, 566-76-7.

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General Method for Isolation of DNA Sequences That Interact with Specific Nuclear Proteins in Chromosomes: Binding of the High Mobility Group Protein HMG-T to a Subset of the Protamine Gene Family†

J. Blanco,‡ J. C. States,‡,§ and G. H. Dixon*

Department of Medical Biochemistry, Faculty of Medicine, Health Sciences Centre, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Received February 22, 1985

ABSTRACT: A general method is described for the isolation of the DNA with which specific nuclear proteins interact in chromosomes. This method is based on the covalent photo-cross-linking of nuclear proteins to the DNA sequences, to which they normally bind, by means of irradiation with UV light and the selective retrieval of specific subsets of protein–DNA adducts by using specific antibodies. The application of this procedure to isolate the DNA sequences with which the trout high mobility group protein (HMG-T) interacts has shown that in trout liver this protein associates specifically with DNA sequences in proximity to a subset of the family of protamine genes but not with the histone or vitellogenin genes. From these observations, it appears that the HMG-T protein may be associated with inactive gene sequences.

It is currently believed that the regulation of gene expression and the conformation of chromatin are mediated by the specific interactions of nuclear proteins with DNA. However, even in the better known cases we have only an imperfect idea of when during development and where in the DNA sequence such associations take place. Some examples of such interactions, recently the focus of active investigation, are the transcriptional complex of *Xenopus* 5S ribosomal RNA genes (Brown, 1984), the binding sites of an HMG-like protein in the satellite DNA of CV-1 monkey cells (Straus & Varshavsky, 1984), the nuclease hypersensitive sites 5′ of heat-shock genes in Drosophila (Elgin, 1981; Wu, 1984), the chicken β-globin gene (Emerson & Felsenfeld, 1984), and the c-myc gene in Burkitt's lymphoma (Siebenlist et al., 1984).

It seems clear that a general procedure for the detection and mapping of the associations between nuclear proteins and specific DNA sequences would be of value in understanding the mechanisms of regulation of gene expression in chromosomes.

It has been possible for some time now to detect the sites of interaction of proteins and DNA in nucleoprotein complexes assembled artificially in vitro (Galas & Schmitz, 1978; Siebenlist et al., 1980; Ross et al., 1978; Ogata & Gilbert, 1977). However, these methods are of limited use in the more complex case of native chromosomes. Very recently, several techniques have been developed that allow the accurate localization of protein binding sites in specific DNA sequences of native chromatin. On the basis of the protective effect that proteins bound to DNA have when chromatin is digested with nucleases or irradiated with UV light, such methods have allowed the localization of nuclear protein binding sites in the nuclease hypersensitive regions 5′ of the heat-shock genes of *Drosophila* (Wu, 1984) and the *lac* repressor binding site in the *lac* operator of *Escherichia coli* DNA (Becker & Wang, 1984), the

† This work has been generously supported by postdoctoral fellowships awarded to J.B. and J.C.S. by the Alberta Heritage Foundation for Medical Research and by operating grants to G.H.D. from the Medical Research Council of Canada.

‡ Postdoctoral fellows of the Alberta Heritage Foundation for Medical Research.

§ Present address: IDR-720, Children's Hospital Research Foundation, Cincinnati, OH 45229.

0006-2960/85/0424-8021$01.50/0