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EVIDENCE FOR TWO CONFORMATIONAL FORMS OF NONHISTONE PROTEIN BA WHICH DIFFER IN THEIR AFFINITY FOR DNA

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Nonhistone protein BA_{free} was purified from the 0.075 M NaCl/0.025 M EDTA/pH 8 extract of whole rat liver nuclei while protein BA_{bound} was isolated from the 0.05 M Na_2HPO_4/8 M urea/1% β-mercaptoethanol/pH 7.6 extract of dehistonized rat liver chromatin. Chromatin associated protein BA_{bound} was able to bind 60% of the [^{3}H] DNA in a nitrocellulose filter binding assay while nucleoplasmic protein BA_{free} showed essentially no DNA binding activity. Circular dichroism analysis of the two forms of protein BA revealed substantial differences in their conformations. Protein BA_{free} was found to have an α-helix content of 41% while protein BA_{bound} displayed a spectrum more typical of unordered or β-turn structures.

Much interest has been focused upon those nonhistone proteins of eukaryotic cell nuclei that can be shown to bind to DNA (1-3). This is, to a large extent, an effort to describe those molecules that have the potential to interact with DNA in a specific manner and modulate the expression of specific genes. Much of the current interest in DNA binding proteins has resulted from their potential role in the maintenance of higher orders of gene structure. One approach has been the study of isolated and highly purified DNA binding proteins which show a correlation in amount with either altered growth state (4) or nutrition (5). One such protein (protein BA) has been isolated from extracts of rat liver chromatin and shown to disappear from the chromatins of growth stimulated hepatocytes (6) and mitogen stimulated lymphocytes (7). This protein was shown to have a binding preference for d(A-T)[1] rich double

[1]Abbreviations used are: BA_{bound}, protein BA isolated from chromatin; BA_{free}, protein BA isolated from the NaCl/EDTA extract of nuclei; d(A-T), deoxyadenine-deoxythymidine; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline (0.01 M sodium phosphate/0.15 M NaCl); PMSF, phenylmethanesulfonyl fluoride; dTTP, deoxythymidine triphosphate.

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stranded DNA (8). In studies using a monospecific antibody probe, protein BA was localized to regions of condensed nuclear and peri-nucleolar chromatin of normal rat liver cells (9).

The present study deals with two conformational forms of protein BA isolated from different nuclear subfractions. Their DNA binding properties are compared in view of their different circular dichroism spectra.

Materials and Methods

Isolation of protein BA_{free} and protein BA_{bound}

Nuclei were isolated from normal rat liver cells by the 0.025 M citric acid method (10). Protein BA_{free} was extracted with 0.075 M NaCl/0.025 M EDTA/1.0 mM PMSF/pH 8, precipitated with 80% of saturated ammonium sulfate, treated batchwise with DEAE-Sephacel in PBS and chromatographed on an Ultrogel AcA 44 column (80x1.6cm) equilibrated with PBS (LKB, Rockville, Md.). Protein BA_{bound} was isolated from dehistonized chromatin by preparative PAGE as described by Catino et al (8).

Analytical Polyacrylamide Gel Electrophoresis

Two-dimensional non-equilibrium pH gradient electrophoresis/SDS gels were modified from the procedure of Hirsch et al (11). The first dimension contained 9 M urea/2% ampholine (pH 3.5-10)/72 mM DTT and was run for 360 V x hr. Apparent pI values were determined by migration relative to pI standards (horse heart cytochrome c/pI 9.4; sperm whale myoglobin, pI 8.0 and 7.7). Bio-Rad low molecular weight standards were run in the SDS second dimension.

Analytical Protein Methods

Amino-terminal determinations were performed by the dansyl-chloride procedure of Weiner et al (12). Carboxy-terminal amino acids were identified by hydrazinolysis (13). Acidic amino acids were blocked by the carbodiimide-nucleophile procedure of Gibson and Anderson using 1-ethyl-3-dimethylaminopropyl carbodiimide (14). Amino acid composition analysis was performed on the Beckman 121MB amino acid analyzer following 5.7 N HCl hydrolysis for 22 hr at 110°C.

DNA Binding Assay

DNA was isolated from normal rat liver nuclei by the method of Sitz et al (15) and nick translated with [^{3}H]dTTP (New England Nuclear, SA = 110 Ci/mmole) as described by Maniatis et al (16). DNA binding was measured in the nitrocellulose filter binding assay of Riggs and Bourgeois (17) as modified by Catino et al (18). Filters were dissolved in 1.0 ml of ethyleneglycol monomethyl ether and counted in a Beckman Model LS-230 liquid scintillation counter.

Circular Dichroism Spectroscopy

Protein samples (20 µg/ml) were placed in a jacketed cylindrical quartz cuvette with a 1.0 cm path and scanned in a Jasco J-500A spectropolarimeter at a sensitivity of 2 m°/cm and a time constant of 2. The data were expressed as the mean residue ellipticity $[\theta]_{MRW}$ in deg cm^2 dmol^{-1}; where $[\theta]_{MRW} = \theta$ [MRW] / 10 x cl, the mean residue weight is 115, l is 1.0 cm and c is the concentration in g/ml.

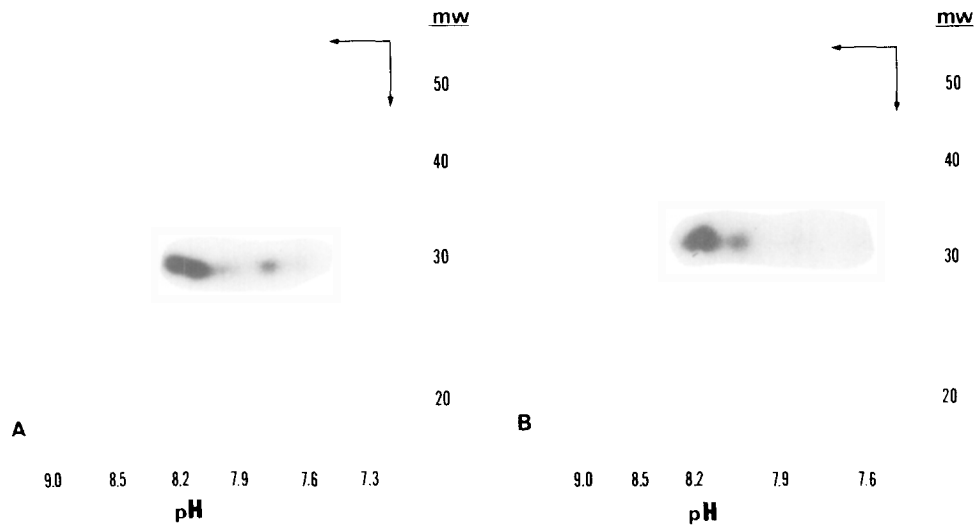

Figure 1 Non-equilibrium pH gradient/ SDS-phosphate two-dimensional PAGE analysis of NaCl/EDTA extracted BA_{free} and chromatin extracted protein BA_{bound}. Highly purified protein BA (50 µg) was lyophilized and dissolved in 2% ampholine (pH 3.5-10)/9 M urea/2 mM DTT prior to loading on the anodic end of the gel. Gels were electrophoresed for 360 V x hr before annealing to a SDS/phosphate second dimension slab gel. Protein BA_{free} is shown in (A) and gel isolated protein BA_{bound} is shown in (B). Gels were stained in 0.25% Coomassie brilliant blue R for 1 hour.

Results

Chemical Comparison of Protein BA_{bound} and Protein BA_{free}

Protein BA_{free} and protein BA_{bound} isolated from nuclei and dehistonized chromatin respectively were analyzed on two-dimensional polyacrylamide gels (Figure 1). In each case protein BA migrated as a series of pI variants at a molecular weight of 31,000. Both samples yielded two major variants at pH 8.0-8.2 in the non-equilibrium pH gradient electrophoresis dimension of the gel. Additional minor variants extended toward the anode to a pH of 7.6.

Upon conjugation with glycine methyl ester in the presence of a water soluble carbodiimide, and repeated analysis on a two-dimensional gel, the variants coalesced into a single major protein spot (Figure 2). This protein spot had a molecular weight of 34,000 and an apparent pI of 9.2. A control sample of protein BA incubated in the absence of water soluble carbodiimide

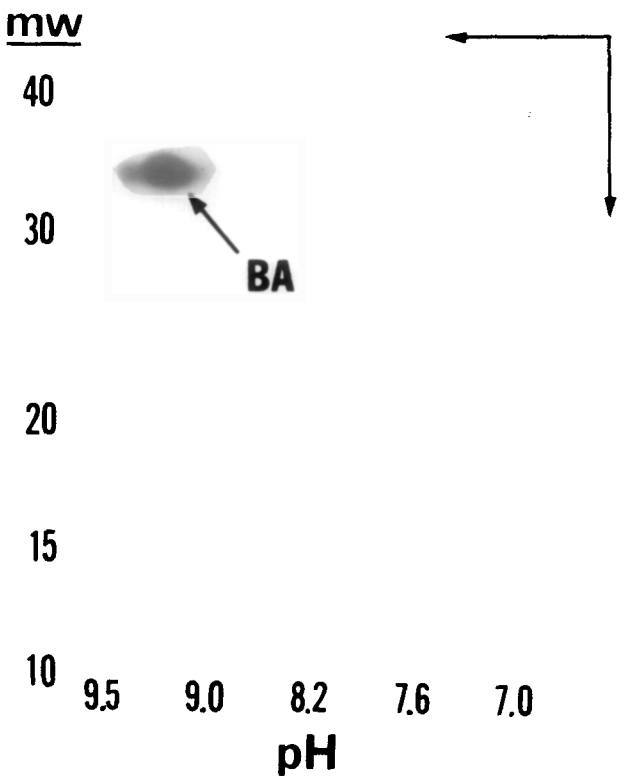

Figure 2 Non-equilibrium pH gradient/SDS two-dimensional PAGE analysis of glycine methyl ester conjugated protein BA_{free}. Protein BA_{free} (75 µg) was conjugated with glycine methyl ester by the water soluble carbodiimide procedure of Gibson and Anderson (15). The conjugated product migrated to an apparent pI of 9.2. See Figure 1 for a description of the running and staining conditions.

resulted in a pattern similar to those shown in Figure 1.

Amino acid analysis and terminal amino acid identification did not reveal any significant chemical differences between protein BA_{bound} and protein BA_{free} (Table I).

DNA Binding Properties of Different Forms of Protein BA

The two forms of protein BA were analyzed for their DNA binding activity in a nitrocellulose filter binding assay (17). Protein BA_{bound} was able to retain 60% of the labeled rat liver DNA in the assay mixture as shown in Figure 3. Analysis of protein BA_{free} at equivalent protein to DNA ratios only resulted in a 2% retention of labeled DNA on the filter. Exposure of protein

TABLE I
AMINO ACID COMPOSITION AND TERMINAL ANALYSIS
OF DIFFERENT FORMS OF PROTEIN BA

Amino Acid	Mole Percent	
	BA_{free}	BA_{bound}
Asp	11.2	9.5
Thr	3.9	4.3
Ser	5.0	6.3
Glu	12.2	11.4
Pro	5.4	6.0
Gly	6.0	8.8
Ala	7.1	5.4
Val	6.0	6.5
Met	1.5	2.2
Ile	5.2	5.0
Leu	11.6	10.0
Tyr	3.5	3.4
Phe	3.9	4.1
Lys	9.7	8.2
His	2.2	2.8
Arg	5.8	6.5
Amino-terminus	blocked	blocked
Carboxy-terminus	Lys	Lys

BA_{free} to the conditions of gel isolation (0.9 M acetic acid or 0.9 M acetic acid/9.0 M urea) did not confer significant DNA binding activity upon it. The binding curves obtained for samples treated in this manner were similar to the one shown for BA_{free} (Fig. 3).

Circular Dichroism Spectra of Nonhistone Proteins BA_{bound} and BA_{free}

The circular dichroism spectra for proteins BA_{bound} and BA_{free} are shown in Figure 4. The spectrum for BA_{free} shows a strong negative peak at 222 nm (-15.5×10^3 deg cm^2 dmol^{-1}) which is indicative of significant α-helix contributions to the protein's conformation. A calculation of the percent α-helix based upon the empirical equation of Chen et al (18) yielded a value of 41 percent α-helix. Protein BA_{bound}, on the other hand, exhibited only a negative shoulder at 222 nm (-5×10^3 deg cm^2 dmol^{-1}) and a strong negative peak at 204 nm (-9.5×10^3 deg cm^2 dmol^{-1}). This type of spectrum suggests that protein BA_{bound} lacks ordered structure or has an extended conformation containing β-turn structures.

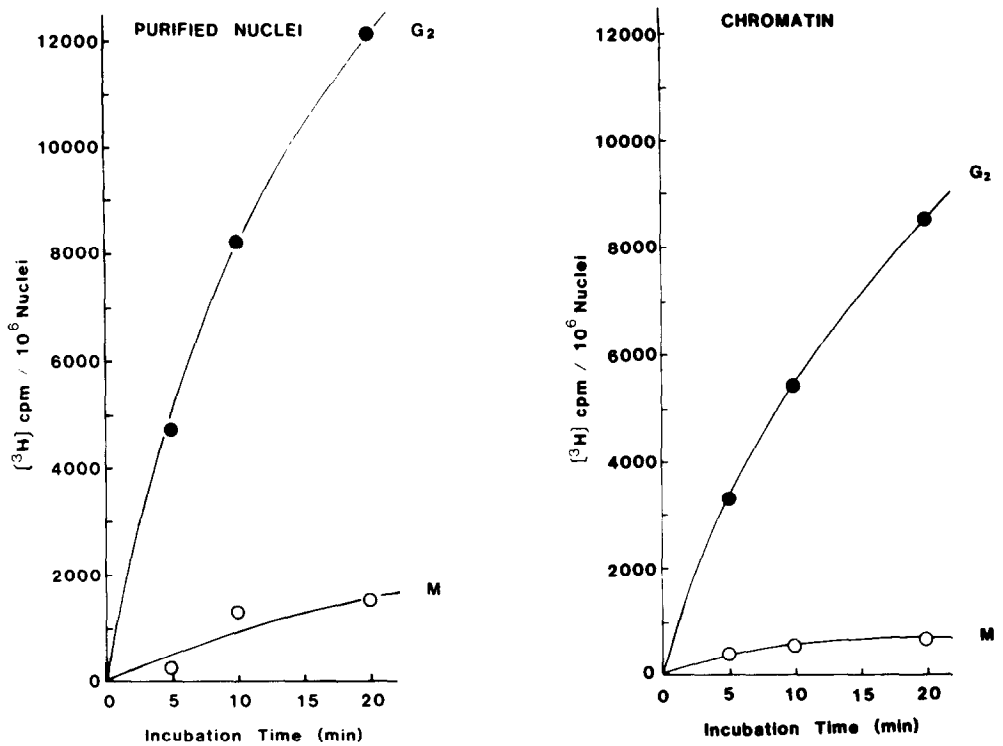

Fig. 2. Incorporation of [^{3}H]UTP into RNA of isolated nuclei (left) and chromatin (right) prepared from plasmodia in G_2-phase (—●—) and metaphase (—○—).

G_2-phase and metaphase at the subnuclear level, namely the preparation from the former was active and that from the latter was inactive just in the same manner as was observed *in vivo* and in the isolated nuclei. Since our preparations preserved *in vivo* properties, the present method seems to be preferable for studying the reguratory mechanism of RNA synthesis in the living cells.

DISCUSSION

Special attentions were given in the isolation of nuclei, especially those at metaphase, because of their extreme fragility. Our method of homogenizing the cells on a sieve was superior to other methods in order to get nuclei with intact morphology. The method was generally applicable to the plasmodia in any stages of the cell cycle including metaphase and anaphase. Triton

Circular dichroism analysis of proteins BA_{free} and BA_{bound} revealed significant differences in their respective amounts of α-helix and non-ordered structure. It is interesting to note that the form of protein BA with the greater contributions from unordered or random structure is the form with DNA binding activity. It is quite possible, however, that a more open or extended conformation is required in order to facilitate its interaction(s) with DNA. Interestingly, the circular dichroism spectrum of protein BA_{bound} resembles the spectrum obtained for gramicidin S which is thought to contain β-turn (reverse turn, β-loop) structures (19). The conformation of protein BA may be critical to its function. Since the roles of these two forms of protein BA may relate to their nuclear compartmentalization, calculations were performed based upon the data in this paper and in a previous report (8). The ratio of BA_{free} to BA_{bound} was found to be 3 to 1. Modulation of protein BA's DNA binding activity through conformational changes may regulate this proportion and thereby aid in the regulation of growth activity.

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