Vol. 90, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 27, 1979 Pages 391-397

BINDING OF HMG-T TO TROUT TESTIS CHROMATIN

LeRoy Kuehl*, Tom Lyness, David C. Watson and Gordon H. Dixon

Division of Medical Biochemistry, The University of Calgary
Calgary, Alberta T2N 1N4, Canada

Received July 17,1979

SUMMARY

When ^{125}I-labeled HMG-T was incubated with trout testis nuclei under conditions of pH and ionic strength approximating those in vivo, most of the radioactivity bound to the chromatin. Most labeled non-nuclear proteins which were tested did not bind. Four large cyanogen bromide fragments of HMG-T each bound, suggesting that HMG-T interacts with chromatin along most of its length. Trout testis chromatin contains two populations of HMG-T molecules which differ in their extractability with NaCl solutions; the ^{125}I-labeled protein equilibrated mainly with the more readily extracted population. HMG-T also bound to nuclease-treated chromatin, an observation with important implications for studies in which nucleases are employed to probe chromatin structure.

INTRODUCTION

The high mobility group (HMG) proteins are a family of well-characterized chromosomal proteins of low molecular weight (< 30,000) which can be extracted from chromatin with dilute salt solutions (e.g., 0.35 M NaCl) and which are soluble in dilute trichloroacetic acid (e.g., 2%) (1,2). They appear to be concentrated in transcriptionally competent regions of the genome (3-5).

Bustin and Neihart, using immunofluorescence techniques, have shown that HMG-1, the major mammalian HMG protein, is present in appreciable concentrations in the cytoplasm as well as in the nucleus in several lines of cultured cells (6). Recently we demonstrated, by microinjection experiments, that the chromatin-bound and cytoplasmic pools of HMG-1 are in equilibrium with one another in HeLa cells and bovine fibroblasts (7). It was of interest to know whether this equilibrium is a dynamic, energy-requiring process, or occurs by simple diffusion and binding to appropriate sites on the chromatin.

* Present address: Department of Biochemistry, University of Utah, 410 Chipeta Way, Salt Lake City, Utah 84108.

0006-291X/79/180391-07$01.00/0

In an effort to answer this question, we have studied the binding of ^{125}I-labeled HMG-T (the trout protein homologous to mammalian HMG-1 (8)) to trout testis chromatin *in vitro*.

MATERIALS AND METHODS

Preparation of nuclei

Rainbow trout testis in the mid stage of development were collected at Mission, British Columbia, and stored at -80° C. The frozen tissue was crushed and homogenized with 4 volumes of 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 7.6, in a Potter-Elvehjem homogenizer with a loosely fitting pestle. The homogenate was filtered through 4 layers of cheesecloth, and the resulting filtrate was centrifuged 10 min at 600 x g. The nuclear pellet was washed once with homogenizing buffer. Nuclei were prepared from fresh trout liver by centrifugation through 2.3 M sucrose (9).

Radiolabeling with ^{125}I

Proteins were iodinated by the lactoperoxidase method as described earlier (7).

Binding studies

The nuclei from 1 g of testis were suspended in 1 ml of homogenizing buffer. To 1 ml of this suspension was added about 10^6 cpm of labeled protein (approximately 0.5 µg) in 2% bovine serum albumin, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.6. The resulting mixture was incubated 30-60 min at 0°. The nuclei were then sedimented, washed 3X with 3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris, pH 7.4 to remove unbound radioactivity, and counted in a gamma counter.

Quantitation of HMG-T

The acid-soluble proteins in the fractions of interest were separated on polyacrylamide gels and stained with Coomassie Brilliant Blue. The amount of HMG-T present was determined from the staining intensity of the appropriate band after construction of a calibration curve relating dye-binding to weight of HMG-T (11).

Preparation of cyanogen bromide fragments

HMG-T was digested with cyanogen bromide as described by Gross (10). The resulting digest was lyophilized and fractionated on a column of Sephadex G75 equilibrated with 20 mM ammonium acetate, pH 5. Seven peptides were eluted and numbered in order of their emergence from the column.

RESULTS AND DISCUSSION

When trace amounts of ^{125}I-labeled HMG-T were incubated at 0° C with trout testis nuclei under conditions of pH and ionic strength approximating those *in vivo*,

Table I

Binding of ^{125}I-labeled proteins to isolated trout testis nuclei

	HMG-T	HMG-1	Soluble cytoplasmic proteins
% of initial counts bound before washing	83	71	18
% of bound counts released during washing			
wash 1	3	3	30
wash 2	1	3	16
wash 3	1	2	11
% of initial counts bound after washing	79	65	8

70-90% of the radioactivity bound to the nuclei and was not released upon washing at low ionic strength (Table I). HMG-1, the calf thymus homolog of HMG-T, behaved similarly. A mixture of cytoplasmic proteins, on the other hand, bound to a considerably lesser degree, and much of the protein which did bind was removed during washing. The non-nuclear proteins myoglobin, bovine serum albumin, and carbonic anhydrase (which has a molecular weight and isoelectric point similar to that of HMG-T) failed to bind to a significant extent (data not shown). Two highly basic non-nuclear proteins did bind: 15% of the label in cytochrome C (pI = 10.0) and 62% of that in lysozyme (pI = 10.8) remained associated with the nuclei after washing. These results are consistent with the hypothesis that the equilibrium between cytoplasmic and chromatin-bound HMG-1 observed *in vivo* is a passive process dependent only upon diffusion of the HMG molecules between the nucleus and the cytoplasm and their binding to appropriate sites on the chromatin.

When labeled HMG-T was incubated with chromatin, instead of nuclei, results similar to those described above were obtained, and we assume that all of our observations, most of which were made with isolated nuclei, reflect the binding of HMG-T to chromatin. Results similar to those described above were also obtained when ^{125}I-HMG-T was incubated with trout liver nuclei, suggesting that the binding is not tissue specific. Binding reached a maximum in one minute, the shortest time investigated. The extent of the reaction was independent of temperature between 0° and 25°, an interval which spans the physiological temperature range for trout. The extent of binding increased as the ionic strength was lowered and decreased as it was raised; standard reaction conditions were chosen to approximate intracellular salt concentrations. Reduced and oxidized HMG-T bound about equally well. In most experiments the reduced form was used because of its greater solubility.

Table II

Binding of ^{125}I-labeled cyanogen bromide fragments of HMG-T to trout testis nuclei

Labeled molecule	% of initial counts bound
intact HMG-T	77
fragment I	42
fragment II	35
fragment III	31
fragment IV	37

Fragment IV contains residues 1-47 of HMG-T. Fragment III extends from residue 48 to about residue 130. Fragment II is about 90 residues in length and is derived from the C-terminal half of the molecule. Fragment I may be composed of fragments III and IV together with a shorter peptide (D.C.W. and G.H.D., unpublished observations).

To determine whether HMG-T contains a specific, well-defined binding site which is responsible for its interaction with chromatin, the molecule was cleaved with cyanogen bromide, and the binding of each of the major fragments thus generated was measured. As shown in Table II, each fragment bound, although not as well as the intact protein. These results suggest that HMG-T interacts with chromatin along much of its length.

By extracting trout testis nuclei with sodium chloride solutions of progressively increasing concentration, two populations of HMG-T can be distinguished: one is released between 0.2 M and 0.4 M NaCl, while the other remains associated with the pellet even after treatment with 0.6 M NaCl (11). As demonstrated in Fig. 1, the ^{125}I-HMG-T which binds to the chromatin in vitro is similar to the more readily extractable population and equilibrates to only a slight extent, if at all, with the more tightly bound fraction.

Most of our experiments were done using tracer amounts of ^{125}I-HMG-T. In an attempt to determine the maximum amount of HMG-T which will bind to a given quantity of chromatin, we prepared ^{125}I-HMG-T of low specific radioactivity and added increasing amounts of this material to constant numbers of nuclei. The results are presented in Fig. 2. Although saturation was not achieved, it is clear that the quantity of added HMG-T which can bind to the isolated nuclei is at least twice as great as the amount of endogenous HMG-T which they contain (the level of endogenous HMG-T is indicated by the dotted line in Fig. 2).

The DNA in transcriptionally competent portions of the genome is known to be preferentially digested by both DN'ase I (12-14) and micrococcal nuclease (15-17). The action of the latter enzyme is, in addition, confined to DNA in the linker regions.

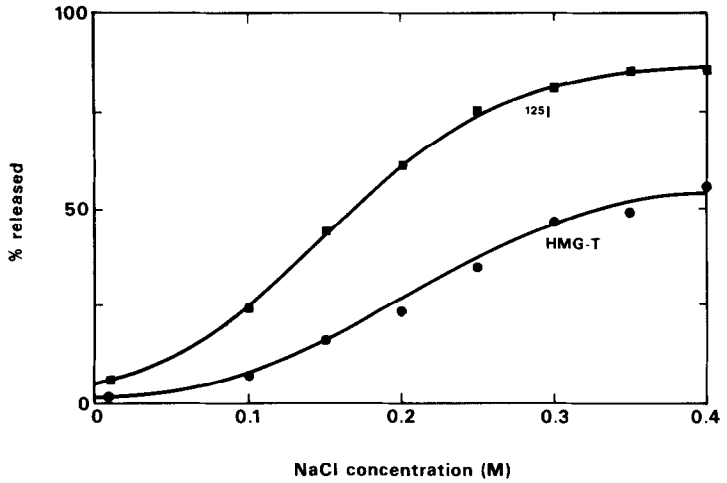

Figure 1 Salt extraction curves for endogenous and ^{125}I-labeled HMG-T. Separate aliquots of nuclei containing bound ^{125}I-HMG-T were extracted for 60 min at 0° with 3.0-ml portions of 3 mM $MgCl_2$, 10 mM Tris, pH 7.4 containing the indicated concentrations of NaCl. The amount of radioactivity (-⊡— ⊡-) and of HMG-T (-O—O-) was determined in each extract and pellet.

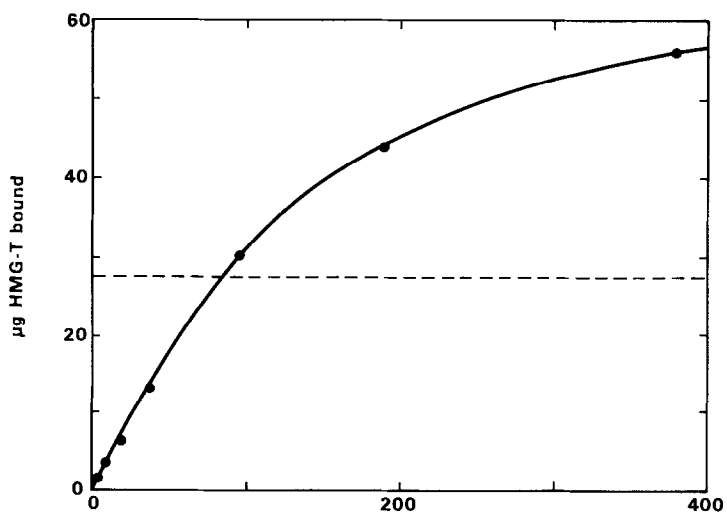

Figure 2 HMG-T binding curve. Each reaction mixture contained one-tenth of the usual quantity of nuclei and the ^{125}I-HMG-T had a specific activity of 18 x 10^3 cpm/µg. Otherwise the binding measurements followed the standard procedure. The dashed line indicates the level of endogenous HMG-T.

Because of this selectivity, these enzymes have been used to determine the position of HMG-T and HMG-1 in the chromatin structure (3,5,18,19). In such experiments it is usually assumed that proteins bound to the nuclease-sensitive portion of the chromatin

Table III

Binding of ^{125}I-HMG-T to nuclease-treated trout testis nuclei

Enzyme	Incubation conditions	% of initial counts bound
None	15 min 0°	85
None	15 min 37°	85
DNase I	15 min 37°	76
None	20 min 0°	82
None	20 min 37°	83
Micrococcal nuclease	20 min 37°	69

For each sample, nuclei from 1 g of testis were suspended in 4 ml of 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris, pH 7.4 (DNase I experiment) or in 8 ml of the same buffer containing 1 mM $CaCl_2$ (micrococcal nuclease experiment). To the appropriate tubes were added 80 µg of DNase I (Worthington, RNase - free, 2300 units/mg) or 200 units of micrococcal nuclease (Worthington, 15,000 units/mg). After incubation as indicated, the nuclei were sedimented and washed once with digestion buffer. Binding was then performed as usual.

will be released following digestion and appear in the supernatant fraction. Our results suggested, however, that these proteins might rebind to the nuclease-resistant pellet. To test this possibility, ^{125}I-HMG-T was incubated with nuclei which had been treated with micrococcal nuclease or DN'ase I. As demonstrated in Table III, ^{125}I-labeled HMG-T bound to the nuclease-resistant regions of chromatin nearly as well as it did to untreated chromatin. Clearly it is not valid to assume that the proteins in nuclease-sensitive regions of the chromatin will appear in the supernatant following digestion, and the results of previous studies must be reinterpreted in the light of this finding.

REFERENCES

1. Johns, E. W., Goodwin, G. H., Walker, J. M., and Sanders, C. (1975) The Structure and Function of Chromatin, Ciba Foundation Symposium 28 (new series) pp. 95-112, Elsevier, Amsterdam.
2. Goodwin, G. H., Walker, J. M., and Johns, E. W. (1979) The Cell Nucleus (Busch, H., ed.) Vol. 6, pp. 181-219, Academic Press, New York.
3. Vidali, G., Boffa, L. C., and Allfrey, V. G. (1977) Cell 12, 409-415.
4. Weisbrod, S., and Weintraub, H. (1979) Proc. Nat. Acad. Sci. US 76, 630-634.
5. Levy W., B., Connor, W., and Dixon, G. H. (1979) J. Biol. Chem. 254, 609-620.
6. Bustin, M., and Neihart, N. K. (1979) Cell 16, 181-189.
7. Rechsteiner, M., and Kuehl, L. (1979) Cell 16, 901-908.
8. Watson, D. C., Peters, E. H., and Dixon, G. H. (1977) Eur. J. Biochem. 74, 53-60.
9. Kuehl, L., and Robison, W. (1979) Biochim. Biophys. Acta, in press.
10. Gross, E. (1967) Methods in Enzymology (Hirs, C. H. W., ed.) Vol. 11, pp. 238-255, Academic Press, New York.

11. Kuehl, L., Lyness, T., Dixon, G. H., and Levy W., B., manuscript in preparation.
12. Weintraub, H., and Groudine, M. (1976) Science 193, 848-856.
13. Garel, A., Zolan, M., and Axel, R. (1977) Proc. Nat. Acad. Sci. US 74, 4867-4871.
14. Levy W., B., and Dixon, G. H. (1977) Nucl. Acids Res. 4, 883-898.
15. Bellard, M., Gannon, F., and Chambon, P. (1977) Cold Spring Harbor Symp. Quant. Biol. 42, 779-791.
16. Bloom, K. S., and Anderson, J. N. (1978) Cell 15, 141-150.
17. Levy W., B., and Dixon, G. H. (1978) Nucl. Acids Res. 5, 4155-4163.
18. Levy W., B., Wong, N. C. W., and Dixon, G. H. (1977) Proc. Nat. Acad. Sci. US 74, 2810-2814.
19. Mathew, C. G. P., Goodwin, G. H., and Johns, E. W. (1979) Nucl. Acids Res. 6, 167-179.