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Carbohydrate modifications of the high mobility group proteins

[glycosylation/poly(ADP-ribose) addition/*Ulex* lectin/nucleosome]

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ABSTRACT **This paper reports the results of numerous biochemical analyses which indicate that the "high mobility group" proteins (HMGs) of mouse and bovine cells are *bona fide* glycoproteins and can, in addition, be modified by poly(ADP-ribose) addition *in vitro*. The sugars *N*-acetylglucosamine, mannose, galactose, glucose, fucose, and one unknown sugar (possibly xylose) have been identified in purified preparations of HMGs 14 and 17. Furthermore, the fucose-specific lectin *Ulex europeus* agglutinin I bound both to the isolated HMGs and to monomer nucleosomes containing HMGs released from "active chromatin" by micrococcal nuclease digestion. Selective alkaline borohydride reductive cleavages of the HMGs suggested that the oligosaccharide prosthetic groups are primarily bound to these proteins by *N*-glycosidic linkages. The unexpected finding that the HMGs contain covalently bound complex carbohydrate moieties allows for a potentially great amount of variability and specificity in these proteins that may have important biological implications.**

Owing to their relatively high concentration within cells (10^5 to 10^6 molecules per nucleus), it seems likely that the nonhistone proteins of the "high mobility group" (HMGs) serve as structural, rather than gene-specific regulatory, components of chromatin (1–4). Nonetheless, these unusual proteins are of considerable interest because they seem to be preferentially associated with "active chromatin" as judged by a number of criteria. For example, studies correlating the selective digestion of "active genes" by DNase I (5) with the preferential release of chromatin proteins strongly suggest that the HMGs are nonrandomly associated with genes in an "active" configuration (6–8). In addition, mild nuclease digestion conditions that selectively release nucleosomes highly enriched in both transcribed DNA sequences (9, 10) and hyperacetylated histones (11, 12) also release nucleosomes enriched in HMGs (6, 9, 12).

Possible structural roles for the HMGs in chromatin do not, however, rule out other concomitant functional roles for these proteins in the nucleus. For example, HMG-14 and HMG-17 have been reported to be able to specifically recognize and interact with nucleosomes from active chromatin and, in so doing, confer on the bound nucleosomes the property of selective DNase I digestion sensitivity (7, 13, 14). Furthermore, both HMG-14 and HMG-17 have been demonstrated to cause partial inhibition of the histone deacetylase enzymes of mouse and calf thymus cells (8). However, all of these and other experiments (15) have failed to elucidate the mechanisms by which HMGs 14 and 17 can specifically recognize and bind to HMG-depleted active nucleosomes (13, 14).

In this context, the results reported here that the HMGs of both mouse and calf thymus cells can be covalently modified by both glycosylation and poly(ADP-ribose) addition are of considerable interest. The finding that the HMGs are nuclear glycoproteins associated with active chromatin allows, at least theoretically, for a whole new unsuspected spectrum of possible molecular interactions and regulatory mechanisms to be investigated at the biochemical level.

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MATERIALS AND METHODS

Cell Line, Culture, and Labeling Conditions. Friend erythroleukemic cells, clone 745A, were maintained and passaged by using described techniques (16). Cells were labeled with radioactive sugar precursors to glycoproteins before isolation of the HMGs as described below. Tritium-labeled sugars (L-[^{3}H]fucose, D-[^{3}H]galactose, D-[^{3}H]mannose, and *N*-acetyl-D-[^{3}H]glucosamine; New England Nuclear) were added directly to the medium of newly subcultured cells at a final concentration of 2.5–5.0 μCi/ml (1 μCi = 3.7×10^4 becquerels) and the cells were grown for 15–19 hr at 37°C before harvesting for protein isolations.

Isolation and Purification of HMGs. HMGs were prepared from isolated Friend cell or calf thymus nuclei as described (8) and then subjected to CM-Sephadex chromatography (17).

"Mini-Gel" Polyacrylamide Electrophoresis. Separation of proteins by electrophoresis in NaDodSO$_4$/polyacrylamide mini-gels was by the method of Matsudaira and Burgess (18). Separation of nucleosomes on nondenaturing ("native") polyacrylamide (4.5%) gels was essentially as described by Bakayev *et al.* (19) with adaptation to the mini-gel system. Transfer of separated proteins or nucleosomes from polyacrylamide gels to nitrocellulose filters was by the procedure of Bowen *et al.* (20).

Glycoprotein and Sugar Determination Methods. (i) *Periodic acid–Schiff* (PAS) staining for glycoproteins in NaDodSO$_4$/polyacrylamide gels was by a modification of the method of Glossman and Neville (21) adapted for the mini-gel system. (ii) *The anthrone reaction for total hexoses* followed the method described by Spiro (22). (iii) *The orcinol/sulfuric acid reaction for total neutral reducing sugars* was performed according to the method described by Gottschalk (23). (iv) *The Dische–Shettles cysteine/sulfuric acid reaction* for the determination of methyl pentoses was used to determine the quantity of fucose in unhydrolyzed HMGs (24). The quantitation of the amount of glycosidically bound sialic acid on HMGs was determined by the *resorcinol reaction* (25). Identification of individual neutral sugars released from purified HMG-14 and -17 proteins by limited acid hydrolysis (under reduced pressure) followed by separation of these sugars from other hydrolytic components by ion-exchange chromatography on sequential columns of Dowex-50 and Dowex-1 was by means of thin-layer chromatography of the sugars on silica gel-G, using as solvent *n*-butyl alcohol/acetone/water (4:5:1) (26). Tentative characterization of the types of glycopeptide bonds present between the HMGs and their carbohydrate moieties was made by the selective alkaline borohydride reduction method for *O*-glycosyl linkages (27) and by

Abbreviations: HMGs, high mobility group proteins; PAS, periodic acid–Schiff.

alkaline hydrolysis followed by selective deamination for the *N*-glycosyl linkages of glycoproteins (28, 29).

Lectin-Binding Studies. A homogeneous preparation of the gorse seed lectin *Ulex europeus* agglutinin I, which specifically reacts with glycoproteins containing fucose residues in their carbohydrate moiety (30), was purchased from Calbiochem. The lectin was chemically iodinated to a specific activity of 1.5×10^7 cpm/μg with ^{125}I-labeled Bolton–Hunter Reagent according to the manufacturer's instructions (New England Nuclear). The iodinated lectin was used in reactions with proteins absorbed either to nitrocellulose filters (see above) or to microtiter plates (Cooke) in a manner analogous to the solid-phase radioimmunoassay procedure described by Romani *et al.* (31).

Other Techniques. For poly(ADP-ribose) incorporation studies, isolated nuclei were incubated with either [^{32}P]NAD$^+$ (32–56 Ci/mmol) or [*adenine*-2,8-^{3}H]NAD$^+$ (around 3–4 Ci/mmol) (New England Nuclear) under described conditions (32). The isolation of monomer nucleosomes by short-term micrococcal nuclease digestion of isolated nuclei followed by sucrose gradient fractionation was by published procedures (33). Fluorography was by the method of Laskey and Mills (34).

RESULTS

HMGs Are Glycoproteins. Fig. 1 shows the results of staining the HMGs and various reference proteins separated by NaDodSO$_4$/polyacrylamide gel electrophoresis with either Coomassie blue (for total proteins) (Fig. 1*A*) or PAS reagent (for glycoproteins) (Fig. 1*B*). It is seen that the calf thymus HMGs (both HMGs 1 and 2 and HMGs 14 and 17), as well as known glycoproteins (ovalbumin and immunoglobulin heavy chain), stained with PAS, whereas proteins that are not glycosylated (the nucleosome "core" histones and reference molecular weight marker proteins) do not (Fig. 1*B*). These results suggest that the HMGs are glycoproteins. Similar results have also been obtained with mouse Friend cell HMGs. However, because poly(ADP-ribose) might also be expected to react with the PAS reagent (32), these results could indicate that the HMGs are modified by addition of poly(ADP-ribose) rather than by being glycosylated.

That the HMGs of mouse Friend erythroleukemia cells are indeed modified by addition of poly(ADP-ribose) is shown in Fig. 2. Fig. 2*A* shows the incorporation of NAD$^+$ radioactivity into acid-insoluble material by isolated nuclei as a function of time of incubation and Fig. 2*B* shows that this incorporated material is sensitive to hydrolysis by dilute alkali treatment, a characteristic of poly(ADP-ribose)-modified proteins (32). Fig. 2*C* indicates that some of the [^{3}H]NAD$^+$ radioactivity is incorporated into the HMGs isolated from these nuclei as shown in the fluorograph of the NaDodSO$_4$ gel separated proteins in lanes 4 and 6. Fig. 2*D* indicates that the [^{3}H]NAD$^+$ radioactivity incorporated into the HMGs (Fig. 2*C*) can also be removed by dilute alkali treatment (lanes 4–6), without degrading these proteins or markedly changing their electrophoretic mobilities (lanes 1–3). However, this ADP-ribosylation is not responsible for the PAS staining noted in Fig. 1, because the HMGs still stain with this reagent after removal of the poly(ADP-ribose) by dilute alkali treatment (data not shown).

To further investigate the nature of the glycosylation of HMGs a number of colorimetric reactions specific for detection of the sugars in glycoproteins were conducted with highly purified preparations of the different proteins. The results of some of these tests are shown in Table 1. From this table it is seen that HMGs 14 and 17, as well as HMGs 1 and 2, react positively in all of the sugar colorimetric assays used (except for the resorcinol reaction for sialic acids), although the relative intensities of the reactions varied for the different proteins.

To verify that the HMGs are indeed true glycoproteins by using an entirely different experimental procedure, isotopically labeled sugar residues that are known to be specific precursors for the biosynthesis of glycoproteins were used to label mouse Friend erythroleukemia cells in culture. After labeling, the HMGs were isolated from the mouse cells and separated by electrophoresis on NaDodSO$_4$/polyacrylamide gels, and the mass bands corresponding to each of the HMGs were cut from the gels and their radioactivities were measured. In the same experiment, the histones from each isotopically labeled cell culture were also isolated in an identical fashion and radioactivity incorporation was measured. The results of these incorporation studies are shown in Table 1. From these data it is seen that whereas the histones fail to incorporate label, all of the HMGs incorporated labeled fucose, galactose, mannose, and *N*-acetylglucosamine.

To determine whether the HMGs contained other sugar residues not apparent from these experiments, highly purified HMGs 14 and 17 were prepared (Fig. 3*A*, lanes 3 and 4) and

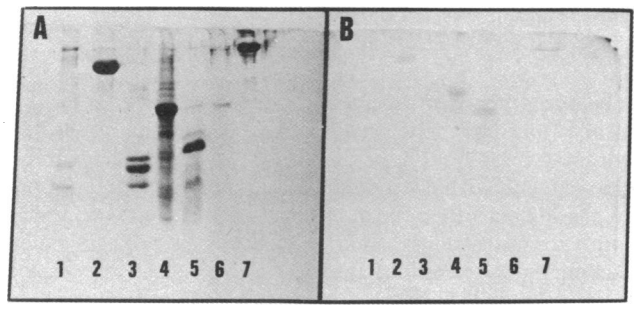

FIG. 1. PAS staining of HMGs. (*A*) Coomassie blue staining of HMGs and reference proteins separated by electrophoresis on a NaDodSO$_4$/polyacrylamide gel. Lanes: 1, molecular weight marker proteins (Sigma); 2, ovalbumin; 3, calf thymus histones; 4, calf thymus HMGs 1 and 2 (crude preparation); 5, calf thymus HMGs 14 and 17 (10% trichloroacetic acid-soluble proteins); 6, crude calf thymus nuclear extract, 5% trichloroacetic acid-soluble and 10% trichloroacetic acid-insoluble proteins; 7, immunoglobulin heavy chains. (*B*) PAS staining of a NaDodSO$_4$/polyacrylamide gel in which the proteins were electrophoresed in parallel with those shown in *A*. Lanes as in *A*. Due to gel swelling during PAS staining the mobilities in *A* and *B* appear to differ.

Table 1. Tests for sugars in proteins

Procedure	HMGs 1 + 2	HMGs 14 + 17	Bovine serum albumin	Ovalbumin	Histones
PAS reaction	++	++	–	++	–
U. europeus I lectin binding	++	++	–	–	–
Anthrone reaction (total hexoses)	+++	++++	–	+	–
Orcinol reaction (reducing sugars)	+	++++	–	++	–
Cysteine/H_2SO_4 reaction	+	++++	–	–	–
Resorcinol (sialic acids)	–	–	–	–	–
Labeled sugars					
[^{3}H]Fucose	+	++++	*	*	–
[^{3}H]Galactose	+	++	*	*	–
[^{3}H]Mannose	+	++	*	*	–
N-Acetyl[^{3}H]-glucosamine	+	+++	*	*	–

* Not determined.

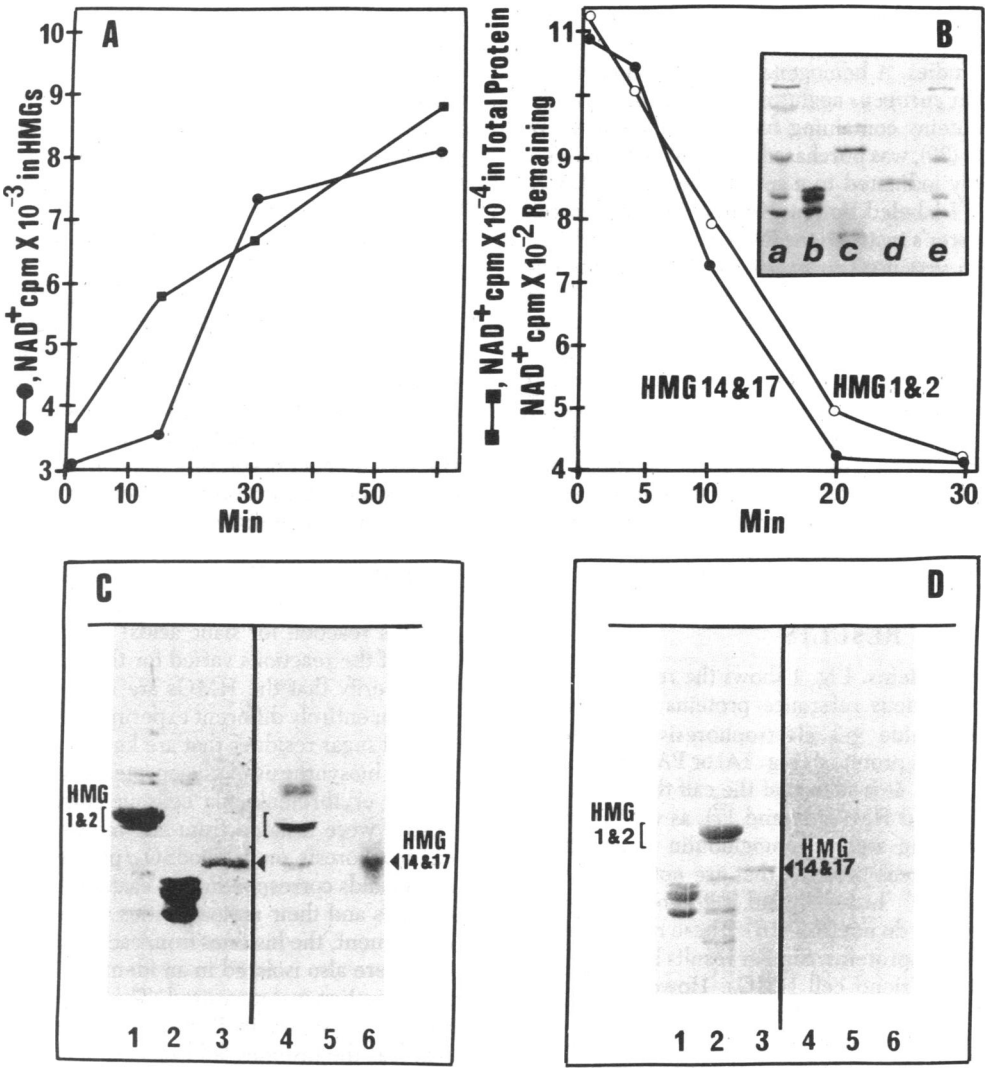

FIG. 2. Addition of poly(ADP-ribose) to the HMG proteins. (*A*) Incorporation of [^{3}H]NAD$^+$ (●) and [^{32}P]NAD$^+$ (■) into acid-insoluble material by isolated Friend cell nuclei. (*B*) Removal of incorporated [^{3}H]NAD$^+$ by dilute alkali hydrolysis (0.1 M NaOH, 37°C) of acid-fractionated HMGs 1 and 2 (○) and HMGs 14 and 17 (●). (*Inset*) NaDodSO$_4$/18% polyacrylamide gel of HMGs after alkali hydrolysis to demonstrate that these proteins were not degraded by this treatment. Lanes: a and e, molecular weight marker proteins; b, calf thymus histones; c, HMGs 1 and 2; d, HMGs 14 and 17. (*C*) NaDodSO$_4$/polyacrylamide gel of proteins labeled *in vitro* with [^{3}H]NAD$^+$. Lanes 1–3, Coomassie blue-stained proteins transferred to nitrocellulose filter. Lanes 4–6, fluorograph of gel shown in lanes 1–3. Lanes 1 and 4, HMGs 1 and 2; lanes 2 and 5, "core" histones; lanes 3 and 6, HMGs 14 and 17. (*D*) Removal of incorporated [^{3}H]NAD$^+$ by dilute alkali hydrolysis. Lanes 1–3, Coomassie blue-stained proteins on nitrocellulose filter; lanes 4–6, fluorograph of lanes 1–3; lanes 1 and 4, core histones; lanes 2 and 5, HMGs 1 and 2; lanes 3 and 6, HMGs 14 and 17.

carbohydrate residues were removed from the proteins by acid hydrolysis. The neutral sugars released by hydrolysis were isolated by Dowex ion-exchange chromatography and the sugars were analyzed by thin-layer chromatography on silica gels (Fig. 3*B*). Fig. 3*A* shows a NaDodSO$_4$ gel of the HMG 14 and 17 preparations used for hydrolysis to demonstrate the degree of purity of the preparations. Fig. 3*B* shows the chromatographically separated and stained neutral sugars from the HMGs (lane 1), along with neutral sugar standards (lane 2). On the right side of this panel is a diagram of the silica plate because some of the fainter sugar spots did not show up well in photographic reproduction. These results confirm the isotope incorporation findings that HMGs 14 and 17 contain the neutral sugars galactose, mannose, and fucose and, in addition, indicate that they also contain glucose and a neutral sugar that had an R_F value similar to xylose. This sugar has not yet been unambiguously identified, however.

The HMG Glycosidic Linkage. The oligosaccharide prosthetic groups of glycoproteins linked *N*-glycosidically from *N*-acetylglucosamine to asparagine in proteins may be distinguished from prosthetic groups linked *O*-glycosidically to the hydroxyl groups of serine or threonine by mild alkaline borohydride reduction, which cleaves the latter prosthetic groups from the peptide via a β-elimination reaction (29). However, under more extreme alkaline conditions followed by a deamination reaction, the *N*-glycosidic linkage can also be selectively cleaved and undegraded oligosaccharide side chains can be released (28). When these two different hydrolytic procedures were applied to the HMGs the mild borohydride hydrolysis failed to remove most of the oligosaccharide side chains, whereas the more extreme hydrolytic conditions released all of the oligosaccharide prosthetic groups (data not shown). These results suggest that a large percentage of the oligosaccharide linkages are of the *N*-glycosidic type.

***Ulex* Lectin-Binding Studies.** The fucose-specific gorse seed lectin *U. europeus* agglutinin I (30) can be used to bind selectively to the HMGs as shown in Fig. 4. In Fig. 4*A* various proteins, including HMGs 1 and 2 and HMGs 14 and 17, have been

dried onto nitrocellulose filters (the "spot" assay) and then allowed to react with ^{125}I-labeled *Ulex* lectin. The autoradiograph of these filters shows that both HMGs 1 and 2 and HMGs 14 and 17 react with the lectin. Similarly, Fig. 4*B* shows that HMGs separated by NaDodSO$_4$ gel electrophoresis, transferred to nitrocellulose, and allowed to react with ^{125}I-labeled lectin also exhibit specific binding. This binding of ^{125}I-labeled lectin to the HMGs can be inhibited by the addition of nonradioactive L-fucose (but not other tested monosaccharides) to the reaction solution.

Fig. 5 shows that ^{125}I-labeled lectin can also bind to monomer nucleosomes containing HMGs and that this binding is inhibited by the addition of nonradioactive fucose (1 mM) in the reaction solution. Monomer nucleosomes were released from calf thymus nuclei by mild micrococcal nuclease digestion and then separated either by sucrose gradient centrifugation (Fig. 5*A*) or by electrophoresis on neutral polyacrylamide gels (Fig. 5*B*). The sucrose gradient was fractionated and aliquots of the fractions were absorbed to the wells of a microtiter plate. Radioactive lectin, either alone or in the presence of added nonradioactive L-fucose, was then allowed to react with the fractions, and bound radioactivity was measured. On the other hand, the nucleosomes separated by gel electrophoresis were transferred to a nitrocellulose filter and then allowed to react with the radioactive lectin. In both cases, it can be seen that in the absence

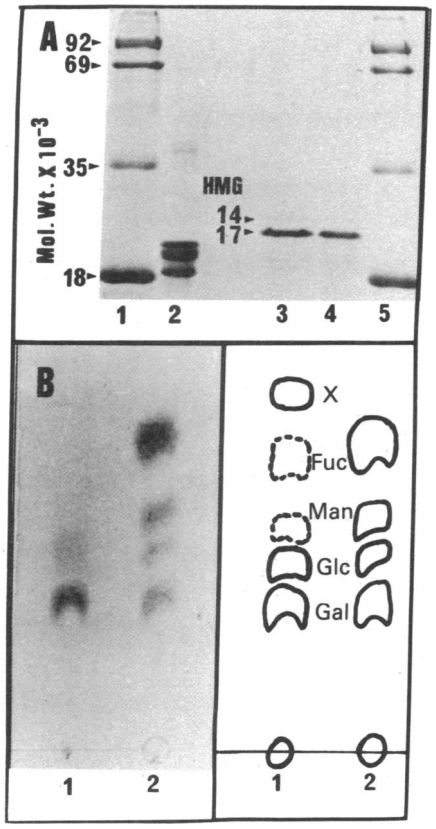

FIG. 3. Determination of sugars found in purified Friend cell HMGs 14 and 17 by thin-layer chromatography. (*A*) Coomassie blue-stained NaDodSO$_4$/polyacrylamide gel of preparations used for the determination of sugar residues. Lanes: 1 and 5, molecular weight markers; 2, calf thymus histones; 3 and 4, purified HMGs 14 and 17 (two different preparations). (*B*) Thin-layer chromatographic separation of sugars isolated from purified HMGs 14 and 17. (*Left*) Lane 1, stained sugars from HMGs 14 and 17; lane 2, standard sugar markers. (*Right*) Diagram of *Left*. Gal, galactose; Glc, glucose; Man, mannose; Fuc, fucose; X, unidentified sugar with the R_F value of xylose.

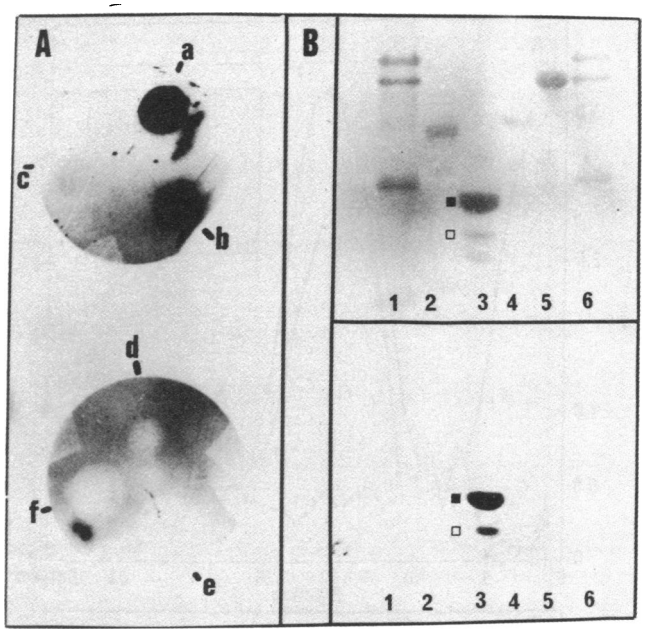

FIG. 4. Binding of the ^{125}I-labeled lectin *U. europeus* agglutinin I (specific for fucose residues) to HMGs. (*A*) "Spot" assay for lectin binding. Spots: a, HMGs 1 and 2; b, HMGs 14 and 17; c, calf thymus histones; d, ovalbumin; e, bovine serum albumin; f, cytochrome *c*. (*B*) (*Upper*) Stained proteins transferred to a nitrocellulose filter after separation of the proteins on a NaDodSO$_4$/polyacrylamide gel. (*Lower*) Autoradiograph of nitrocellulose filter shown in *Upper* after reaction with lectin. Lanes: 1 and 6, molecular weight markers; 2, ovalbumin; 3, HMGs (■, HMGs 1 and 2; □, HMGs 14 and 17); 4, immunoglobulin heavy chain; 5, bovine serum albumin.

of free L-fucose the nucleosomes bind *Ulex* lectin. That the monomer nucleosomes also contain HMGs is shown by the *Inset* in Fig. 5*A*, which shows the NaDodSO$_4$ gel electrophoretic profile of HMGs extracted by selective acid treatment of pooled fractions of the monomer nucleosomes.

DISCUSSION

The results reported here demonstrate that the HMGs of both calf thymus and mouse Friend erythroleukemic cells can be covalently linked to either poly(ADP-ribose) or complex oligosaccharide moieties. The apparent *N*-glycosidic linkage of the oligosaccharide side chains containing, at least for HMGs 14 and 17, the sugars galactose, mannose, glucose, fucose, *N*-acetylglucosamine, and possibly xylose definitely places these HMGs into the class of "complex glycoproteins" as defined by Gottschalk (23). Furthermore, from the known amino acid sequence data of the calf thymus proteins (2–4) it is apparent that each of the major HMGs contains one or more of the tripeptide sequences usually associated with *N*-glycosidically linked glycoproteins (29, 35), although the actual amino acid residues linked to the oligosaccharide moieties remain to be determined.

Whereas the presence of poly(ADP-ribose) on the HMGs was not entirely surprising, given that many other nuclear proteins, such as the histones, can be modified in this way (32), the finding that the HMGs are covalently bonded to appreciable amounts of sugar residues was unexpected. Nonetheless, previous workers have reported that plant lectins can bind to unidentified nonhistone chromatin proteins within nuclei (36) or in isolated nucleosomes (37). The findings reported here demonstrate, on the other hand, that the specific nonhistone HMGs that are associated with active chromatin are true glycoproteins and suggest, furthermore, that sugar-specific lectins can be used as biochemical probes to investigate this chromatin.

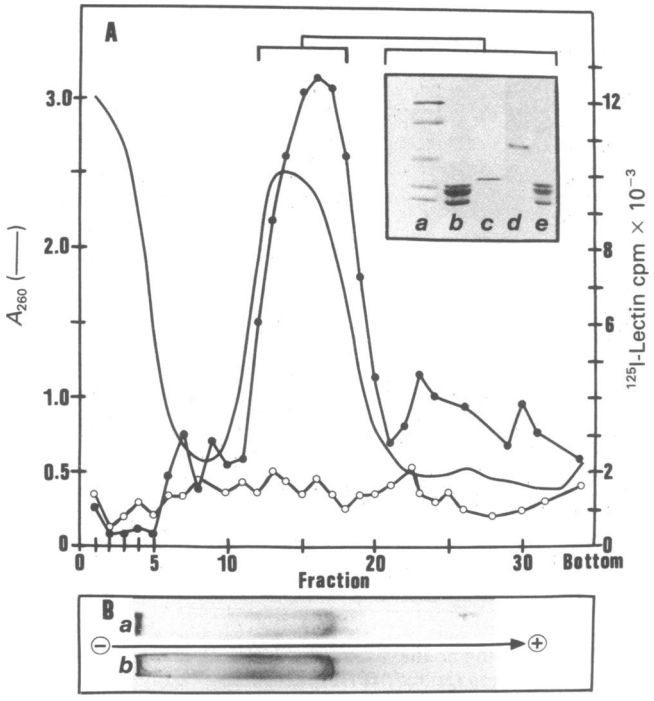

FIG. 5. Binding of ^{125}I-labeled *Ulex* lectin to nucleosomes. (*A*) Separation of nucleosomes from a short-time micrococcal nuclease digestion of Friend cell nuclei by centrifugation on a 10–30% sucrose gradient. Fractions from the gradient were allowed to react with ^{125}I-labeled lectin. ●, cpm of lectin bound to fractions; ○, cpm bound in the presence of competing L-fucose (1 mM). ——, Absorbance at 260 nm. (*Inset*) Bracketed fractions were pooled, and histones (lane b), HMGs 14 and 17 (lane c), and HMGs 1 and 2 (lane d) were extracted. The extracted proteins were separated by electrophoresis on a NaDodSO$_4$/18% polyacrylamide gel along with standard molecular weight markers (lane a) and marker calf thymus histones (lane e). (*B*) Monomer nucleosomes separated by electrophoresis on a nondenaturing polyacrylamide gel and transferred to a nitrocellulose strip and stained with Coomassie blue (lane b). The material on the nitrocellulose strip was allowed to react with ^{125}I-labeled lectin and then subjected to autoradiography (lane a).

The role or function of glycosidic modification of the HMGs is unknown. However, of the many postulated functions for protein glycosylation (23, 29, 35) perhaps the most interesting in terms of HMGs 14 and 17 has to do with the possibility that the glycosylation might be involved either in specific intracellular molecular recognitions (38) or in the "routing" of these glycoproteins from their sites of synthesis and modification to their sites of specific cellular function in a manner analogous to that of certain lysosomal glycoproteins (39). Furthermore, preliminary experiments suggest that there may be heterogeneity in the types of carbohydrate moieties that different HMGs contain, thus potentially allowing for a great amount of variability and specificity in the HMGs due to this modification. Such variability might have important biological functions.

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