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ANALYSIS OF NUCLEOPROTEINS BY DIRECT INJECTION OF DISSOLVED NUCLEI OR CHROMOSOMES INTO A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM*

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SUMMARY

This work describes the development of a method for fractionating the total nuclear proteins of Chinese hamster cells (line CHO) by high-performance liquid chromatography of whole nuclei or chromosomes. Nuclei or chromosomes were dissolved for 2 h in 6 *M* guanidine hydrochloride containing 0.2% trifluoroacetic acid (TFA). Residual particulates were removed by centrifugation, and a sample of the solution was passed through a Bio-Sil TSK guard column, followed by a Bio-Sil TSK 400 column, equilibrated with water containing 0.2% TFA. These columns fractionated the sample by adsorbing the DNA, passing the proteins near the exclusion limit, passing the guanidine at the inclusion limit, and passing an unidentified non-protein/non-DNA material after the inclusion limit. The protein peaks from the TSK columns were collected directly on a μBondapak CN reversed-phase column. The protein fractions were then eluted from the CN column with an acetonitrile gradient containing 0.2% TFA. Three other reversed-phase columns were examined for use with the TSK columns. The μBondapak C_{18} Radial-Pak column produced the best resolution of histone variants, while the Zorbax C_8 column produced a better resolution of the non-histone proteins. The Nova-Pak C_{18} Radial-Pak column was found to be unsatisfactory for both classes of nucleoproteins.

INTRODUCTION

The regulation of genetic activity is thought to occur as a result of interactions between proteins and DNA in the cell nucleus. Studies of the role of proteins in this regulation usually involve treating cells with genetically modulating agents, (hormones, drugs, toxic agents, carcinogens, radiation, etc.) and then measuring changes in the nucleoproteins of isolated chromatin. To do this, nuclei are isolated from the cells, chromatin is prepared from the nuclei, proteins are extracted from the chromatin, and the proteins are fractionated (usually by some electrophoretic method). There can be problems with this procedure, the most serious one being quantification

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of the changes in the proteins of the nucleus following treatment. This arises from the fact that, with the exception of the nucleosomal core histones, the interaction of the proteins with the DNA may not be strong enough to keep the proteins of interest attached to the DNA through the chromatin isolation procedure so that they can be accurately quantified in subsequent analytical steps. Additionally, these procedures are frequently laborious, time-consuming, and do not lend themselves well to preparative recovery of the purified proteins for further chemical, structural, and biological function analysis.

As a result of these concerns, we sought to develop a procedure which fractionates and quantifies the total protein complement of the cell nucleus directly from isolated nuclei in one step. In our laboratory, we had recently developed a high-performance liquid chromatographic (HPLC) method for the analysis of histones and non-histone HMG proteins isolated from chromatin[1–4]. This HPLC method produced quantitative recovery of histones from the HPLC column and was suitable for preparative use due to the volatility of the chromatographic solvents used. Thus, it was felt that this HPLC method whould provide a good basis for the analysis of nuclei if a method could be found for applying whole nuclei directly to the column. Henderson *et al.*[5] had found that whole Gross leukemia RNA virus could be disrupted in 6 *M* guanidine hydrochloride (Gu · HCl) containing trifluoroacetic acid (TFA) and then applied directly to a reversed-phase HPLC column to produce a chromatogram of viral structural proteins in an acetonitrile gradient. Because of the similarities of that viral HPLC system with our histone HPLC system, we attempted to apply nuclei dissolved in Gu · HCl directly to our HPLC column. Unfortunately, the large amounts of DNA and the Gu · HCl in the sample of nuclei interfered with the analysis. In this report, we describe the problems encountered, present the methods we found for their elimination, and discuss the development of a one-step process for the protein analysis of whole nuclei and chromosomes.

EXPERIMENTAL

Preparation of nuclei, chromosomes, and histones

Chinese hamster cells (line CHO) were grown exponentially in suspension culture, as described by Tobey *et al.*[6]. For radiolabeling of DNA, 3 μCi of [methyl-^{14}C]thymidine (specific activity 50 mCi/mmole, New England Nuclear) was added per liter of culture medium 24 h prior to harvest of cells. For radiolabeling of protein, 50 μCi of L-[4,5-$^{3}H_2$]lysine (specific activity 60 Ci/mmole, Schwartz/Mann) or 50 μCi of [^{14}C]lysine (specific activity 312 mCi/mmole, Schwartz/Mann) was added per liter of culture medium 24 h prior to harvest of cells.

For the preparation of histones, chromatin was prepared from whole cell homogenates and acid-soluble proteins were extracted from this chromatin with 0.2 *M* sulfuric acid. Whole histones were recovered by precipitation with acetone and lyophilized to dryness, as described by Gurley *et al.*[1].

Nuclei were prepared from 1-l suspension cultures containing 300 000 cells/ml by using the non-ionic detergent Nonidet P-40 (Shell) and the ionic detergent sodium deoxycholate, as described by Gurley *et al.*[7]. The nuclei were frozen at −70°C until used.

Chromosomes were prepared from 75–85% confluent monolayer cultures of

CHO cells, grown in T150 culture flasks. Fifteen such cultures were treated for 5 h with Colcemid (0.1 μg/ml) to arrest cells in mitosis. The mitotic cells were then removed from the monolayer by shaking, producing a population with a mitotic index of 0.97. Chromosomes were prepared from these cells by a modification of the hypotonic potassium chloride/Triton X-100/RNase/propidium iodide method of Aten *et al.*[8], as previously described by Cram *et al.*[9]. This procedure produced $3.2 \cdot 10^8$ chromosomes (the number equivalent to $5.1 \cdot 10^7$ nuclei). Such chromosomes are suitable for flow cytometry sorting and flow karyotype analysis by propidium iodide fluorescence. It is anticipated that the HPLC analysis procedures described in this report will be used in the future to analyze such sorted chromosomes. The chromosomes were frozen at −70°C until used. In one case, nuclei were prepared by the same procedure as that described for chromosomes. These nuclei were mixed with chromosomes and frozen until used.

Dissolution of nuclei and chromosomes

Frozen nuclei from 1-l cultures ($3 \cdot 10^8$ nuclei) or $3 \cdot 10^8$ chromosomes were thawed and dissolved in 1–10 ml of 6 *M* Gu · HCl, containing 0.2% TFA, for 2 h with occasional stirring with a glass rod. The small amount of insoluble material remaining was then removed by centrifuging the sample 15 min at 8000 *g*. The supernatant fluid was removed, and varying quantities (10–1000 μl) were injected directly into the HPLC system. The remaining sample was frozen at −70°C for use in other experiments.

Reversed-phase HPLC columns

Reversed-phase HPLC was performed on several different columns manufactured by Waters Assoc. (Milford, MA, U.S.A.): (1) μBondapak CN column (30 cm × 3.9 mm I.D. steel column containing cyanopropylsilane bonded to an end-capped 10-μm irregular particle size porous silica packing); (2) μBondapak C_{18} Radial-Pak column (10 cm × 8 mm I.D. plastic cartridge containing octadecylsilane bonded to an end-capped 10-μm irregular particle size porous silica packing); (3) Nova-Pak C_{18} Radial-Pak column (10 cm × 8 mm I.D. plastic cartridge containing octadecylsilane bonded to an end-capped 4-μm uniform spherical particle size porous silica packing). The μBondapak C_{18} and Nova-Pak C_{18} Radial-Pak columns were contained in a Waters Z-Module, designed for radial compression of the flexible-walled plastic cartridges to produce minimum void volume and maximum efficiency[10]. A reversed-phase Zorbax 150-C_8 column, manufactured by Du Pont Instruments (Wilmington, DE, U.S.A.), was also used. This column (8 cm × 6.2 mm I.D. steel column containing octasilane bonded to an end-capped 5–7 μm spherical particle size porous microparticulate silica packing) was equipped with a 0.5-μm column protection prefilter.

Gel filtration HPLC columns

HPLC gel filtration chromatography was performed on a series of Bio-Sil TSK columns manufactured by Bio-Rad Labs. (Richmond, CA, U.S.A.). These columns are packed with a rigid silica-based gel filtration material having a hydrophilic bonded phase to eliminate residual silanol activity and to reduce hydrophobic absorption. Three different columns were used (separate, or in a series): (1) Bio-Sil TSK guard

column (75 mm × 7.5 mm I.D. steel column); (2) Bio-Sil TSK-400 column (300 mm × 7.5 mm I.D. steel column, packed with 13-μm particles); (3) Bio-Sil TSK-250 column (300 mm × 7.5 mm I.D. steel column, packed with 10-μm particles). The Bio-Sil TSK guard column was packed with the same material as the Bio-Sil TSK-250 column, except that the mesh size was more variable. The TSK-400 column had a protein molecular weight range of 5000–1 000 000, while that of the TSK-250 column was 1000–300 000.

HPLC mobile phase

In both gel filtration and reversed-phase chromatography systems the solvents were delivered to the columns by a Waters Model 6000A solvent delivery system. The reversed-phase columns were equilibrated with either water containing 0.2% TFA or with 6 *M* Gu · HCl containing 0.2% TFA, depending on the experiment. The gel filtration columns, or tandem arrays of gel filtration and reversed-phase columns were equilibrated with the appropriate solvent (water containing 0.2% TFA or water–acetonitrile, containing 0.2% TFA). The samples, varying in size from 10 μl to 1000 μl (histones dissolved in water containing 0.2% TFA and nuclei or chromosomes dissolved in 6 *M* Gu · HCl containing 0.2% TFA) were injected into the HPLC system with a Waters Model U6K injector, fitted with 2-ml sample loop.

Proteins were eluted from the gel filtration columns isocratically, as indicated in each experiment. Elution from the reversed-phase columns was accomplished with linear gradients of water–acetonitrile containing 0.2% TFA. The gradients progressed from water containing 0.2% TFA to acetonitrile containing 0.2% TFA and were generated by three pumps under the control of a Waters Model 720 system controller: pump A supplied water containing 0.2% TFA, pump B supplied water–acetonitrile (50:50) containing 0.2% TFA, and pump C supplied acetonitrile containing 0.2% TFA. The use of 50% acetonitrile is preferred during the earlier part of the gradient, because fewer solvent mixing anomalies are observed at low acetonitrile concentrations when prediluted solvents are used[4].

Methods of analysis for HPLC effluents

Proteins eluted from the columns were detected by UV absorption at 206 nm with a Waters Lambda-Max Model 481 flow spectrophotometer. In some experiments, the column effluent was collected in 1-ml fractions. These fractions were then either mixed with 12 ml Formular 963 scintillation cocktail (New England Nuclear) for the determination of their radioactivity in a Tri-Carb Model 3320 liquid scintillation spectrometer, or they were pooled according to designated chromatographic peaks, frozen, lyophilized, and then used for electrophoretic analysis. Electrophoresis of HPLC fractions from nuclei or chromosomes was performed by the acid–urea gel system of Panyim and Chalkley[11]. The specific conditions used in this laboratory for the acid–urea system (2.5 *M* urea–0.9 *M* acetic acid–15% polyacrylamide gels) have been previously described by Gurley *et al.*[12].

RESULTS

Chromatography of nucleoproteins on μBondapak CN columns equilibrated with water containing 0.2% TFA

In our laboratory we have developed an HPLC system for the chromatography of histones[1–4] which resolves whole histone preparations into the five histone types (H1, H2A, H2B, H3, and H4), as shown in Fig. 1A. In an attempt to extend the use of this system to the chromatography of the total protein complement of nuclei, nuclei were dissolved in 6 *M* Gu · HCl in order to disrupt all nucleic acid–protein complexes[5]. Following clarification by centrifugation, the solution was loaded directly on the column. The histones and other proteins were eluted as shown in Fig. 1B. However, a broad peak of unidentified substance (US) was eluted just before H1, obscuring this area of the chromatogram, and large amounts of material were eluted at the beginning of the chromatogram (Fig. 1B). The amount of this latter material increased with each additional run until it filled the entire chromatogram. This material was suspected to be DNA which accumulated by adsorption on the column. To confirm this, the 6 *M* Gu · HCl sample of dissolved nuclei was diluted to 0.6 *M* Gu · HCl. This reduction in the solubilizing activity of the Gu · HCl, combined with the acidic pH of the 0.2% TFA resulted in a precipitation of the DNA. The DNA was removed by centrifugation, and the solution containing the proteins was injected

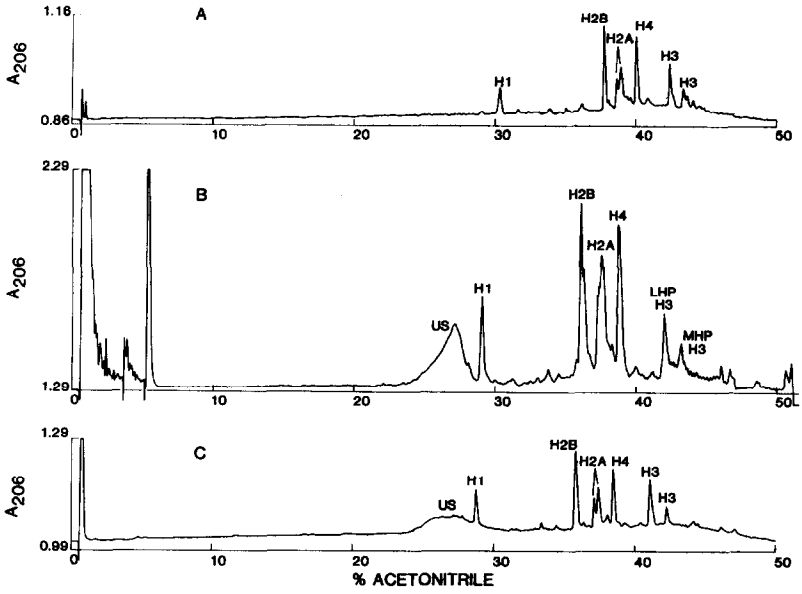

Fig. 1. HPLC of CHO nucleoproteins on a μBondapak CN column equilibrated with water containing 0.2% TFA. A, Injection of 250 μg of whole histone dissolved in 100 μl water containing 0.2% TFA. B, Injection of 8.5 · 10^7 nuclei, dissolved in 1 ml 6 *M* Gu · HCl containing 0.2% TFA. C, Injection of 3 · 10^7 nuclei, dissolved in 0.1 ml of 6 *M* Gu · HCl containing 0.2% TFA and then diluted with 1 ml of water containing 0.2% TFA to precipitate DNA. The DNA was removed by centrifugation and the supernatant fluid (1.1 ml) was injected. Following injection of the above samples, all three columns were eluted with a 0–50% linear gradient of acetonitrile containing 0.2% TFA in 300 min at 1 ml/min. LHP and MHP refers to less-hydrophobic and more-hydrophobic variants, respectively.

into the HPLC system (Fig. 1C). The acid-soluble histones were eluted along with the US peak. A single initial peak was eluted in the void volumn. This was suspected to be Gu · HCl, but the large amount of interfering DNA material was absent. Unfortunately, there was little indication that non-histone proteins were present in this chromatogram. It is probable that these proteins were acid-precipitated along with the DNA when the 6 *M* Gu · HCl sample was diluted.

Chromatography of nucleoproteins on μBondapak CN columns equilibrated with 6 M Gu · HCl containing 0.2% TFA

It was thought that perhaps DNA might not be adsorbed on the column if the column was equilibrated with Gu · HCl. Therefore, nuclei were dissolved in 6 *M* Gu · HCl containing 0.2% TFA and loaded onto a column equilibrated with the same solution. The Gu · HCl was then washed out of the column with water containing 0.2% TFA, and the proteins were eluted with an acetonitrile gradient (Fig. 2A). A peak of material was eluted almost immediately. At 20–35% acetonitrile the US fraction was eluted, but no H1 was observed to emerge at 29% acetonitrile. It was not known if the H1 was missing or obscured by the elution of US. Comparison of

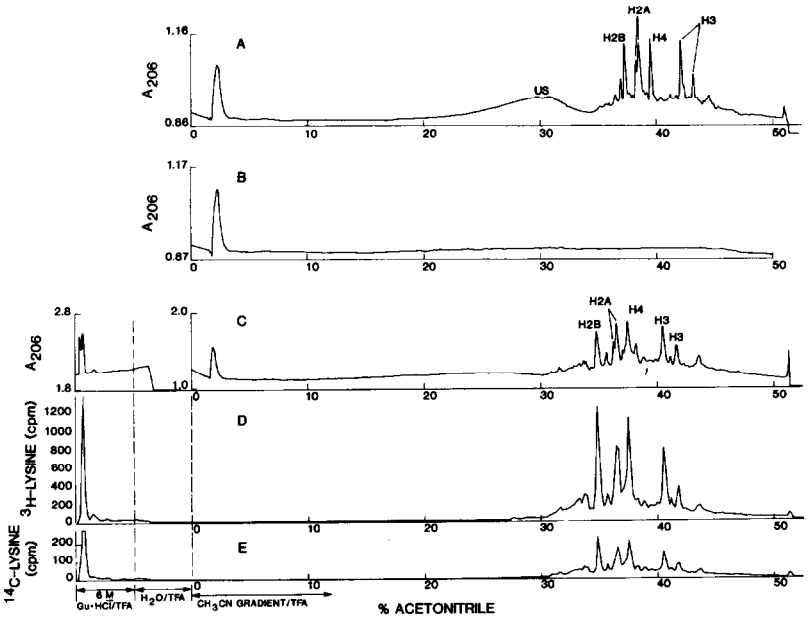

Fig. 2. HPLC of CHO nuclei and chromosomes on a μBondapak CN column equilibrated with 6 *M* Gu · HCl containing 0.2% TFA. A, Injection of 2 · 10^7 nuclei, dissolved in 500 μl 6 *M* Gu · HCl containing 0.2% TFA. B, System control: injection of 500 μl 6 *M* Gu · HCl containing 0.2% TFA containing no nuclei. C, Injection of a mixture of 2 · 10^7 [^{3}H]lysine labeled chromosomes and 3 · 10^7 [^{14}C]lysine labeled nuclei, dissolved in 600 μl 6 *M* Gu · HCl containing 0.2% TFA. D, Radioactivity profile of the [^{3}H]lysine labeled chromosomes in the chromosome–nuclei mixture above. E, Radioactivity profile of the [^{14}C]lysine labeled nuclei in the chromosome–nuclei mixture above. Following injection of the above samples all the columns were eluted with 6 *M* Gu · HCl containing 0.2% TFA for 30 min, followed by water containing 0.2% TFA for 30 min. The proteins were then eluted with a 0–50% linear gradient of acetonitrile, containing 0.2% TFA in 300 min, at 1 ml/min.

this chromatogram with Fig. 1A indicated that some non-histone proteins were eluted among the nucleosomal core histones (H2B, H2A, H4 and H3).

A blank control elution of an unloaded column which had been equilibrated with 6 *M* Gu · HCl containing 0.2% TFA, indicated that the initial peak was present, but the US fraction was not (Fig. 2B). Thus, the US fraction is obtained from the nuclei, but the initial peak is a product of the HPLC system. Since the column in Fig. 2B was washed free of Gu · HCl before acetonitrile elution began, this peak must represent either guanidine or a guanidine–TFA complex which is adsorbed on the column during equilibration and is dissociated at low acetonitrile concentrations.

While the presence of non-histone proteins in Fig. 2A suggested that some non-histone proteins of the nucleus were being recovered by this procedure, the absence of H1 indicated that some proteins were also being lost. To confirm this, [^{14}C]lysine-labeled nuclei were dissolved and subjected to HPLC (Fig. 2C and 2E). Elution of ^{14}C-proteins (Fig. 2E) was confined to two areas of the chromatogram: (1) the void volume during loading in the presence of 6 *M* Gu · HCl, and (2) the protein elution region at 30–45% acetonitrile. From this, it is concluded that no protein is associated with the initial guanidine elution peak at 1–2% acetonitrile nor with the US fraction. No ^{14}C-labeled H1 was observed at 29% acetonitrile, confirming our conclusion that this very lysine-rich histone H1 is not adsorbed on the column when it is equilibrated with Gu · HCl.

To determine whether Gu · HCl prevented the adsorption of DNA on the column, [^{14}C]thymidine-labeled nuclei were dissolved and loaded onto a column equilibrated with the same solution. The [^{14}C]DNA was eluted in the void volumn during loading (Fig. 3A). The column was then equilibrated with water containing 0.2% TFA. No further DNA elution was detected during this equilibration, nor during the subsequent protein elution with the acetonitrile gradient. This demonstrated that the guanidine, US, and protein peaks, were all free of DNA.

In this experiment, a large number of nuclei were loaded on the column to facilitate preparative recovery of the proteins. The fractions marked in Fig. 3A were lyophilized and subjected to electrophoresis (Fig. 3B). Electrophoresis identified the histone proteins were adsorbed on the column and then eluted (peaks A–H in Fig. 3B). This confirmed our conclusions from Fig. 2E that H1 is not adsorbed on the μBondapak CN column equilibrated with Gu · HCl. However, a number of non-histone proteins was adsorbed on the column and then eluted (peaks A–H in Fig. 3A). These proteins are not normally seen in histones prepared from chromatin (Fig. 1A). In the electropherogram (Fig. 3B) the proteins A–H all appear to contain some histone fraction. It is not yet known whether this represents a naturally occurring complex between histone and non-histone proteins or a sample contamination inherent in the HPLC system.

Chromatography of chromosome proteins on μBondapak CN columns equilibrated with 6 M Gu · HCl containing 0.2% TFA

Because it is anticipated that the HPLC procedures developed in this report will be used to analyze sorted chromosomes as well as isolated nuclei, chromosomes were prepared which contained propidium iodide, a fluorochrome commonly used to facilitate chromosome sorting by flow cytometry[9]. These chromosomes were dissolved in 6 *M* Gu · HCl containing 0.2% TFA, loaded on a μBondapak CN column

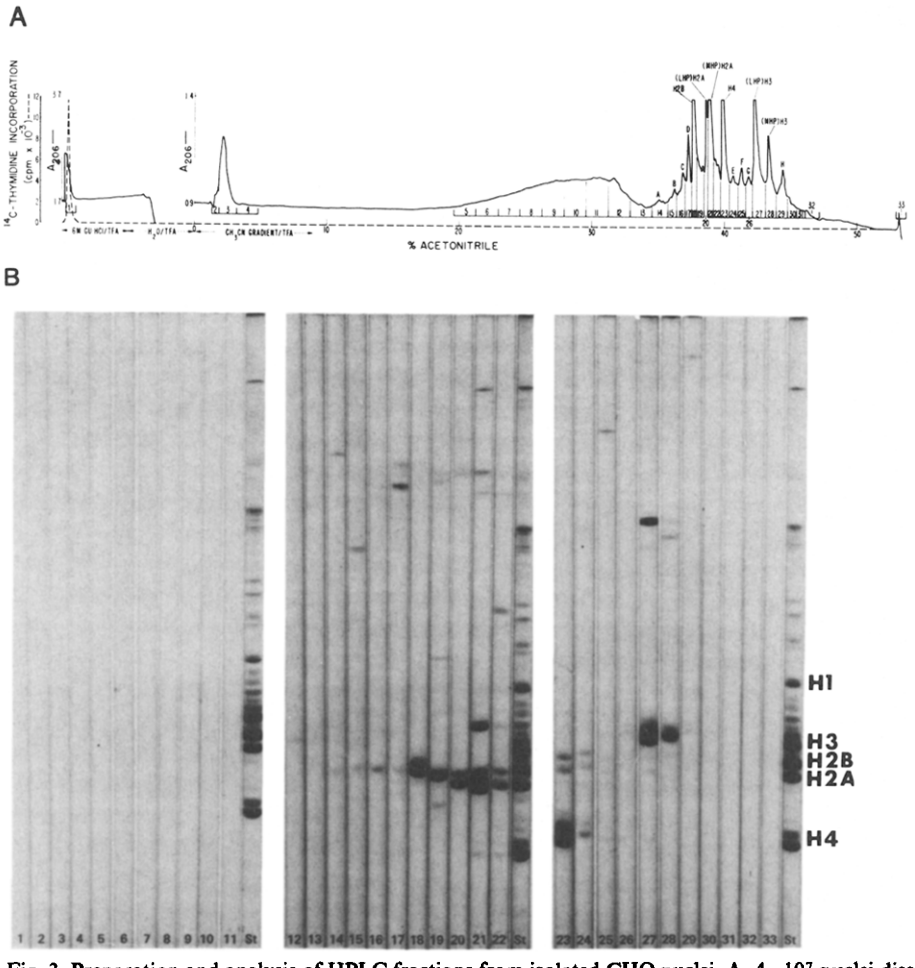

Fig. 3. Preparation and analysis of HPLC fractions from isolated CHO nuclei. A, $4 \cdot 10^7$ nuclei dissolved in 1000 μl 6 *M* Gu · HCl containing 0.2% TFA were injected into a μBondapak CN column, equilibrated with 6 *M* Gu · HCl containing 0.2% TFA. The column was eluted as shown in Fig. 2. Effluent fractions were collected as indicated in this figure and lyophilized. B, The HPLC fractions indicated were subjected to acid–urea polyacrylamide gel electrophoresis along with whole histone preparations, which were used as identification standards (St).

equilibrated with the same solvent, and subjected to HPLC to determine whether the propidium iodide would interfere with the HPLC system, with the protein detection at 206 nm, or with protein retention times on the column (Fig. 4A). Chromosomes produced no significant US peak. Chromosomal H1 was missing, just as it was when nuclei were chromatographed in this system, but many proteins in addition to the nucleosomal core histones were present. Fractions collected from this column were lyophilized and subjected to electrophoresis (Fig. 4B). From this, the histones were identified as marked in Fig. 4A. Several non-histone proteins were eluted before histone H2B. Of these, fraction 10 from chromosomes (Fig. 4) had the same electrophoretic pattern as peak C from nuclei (fraction 15, Fig. 3). From these comparisons

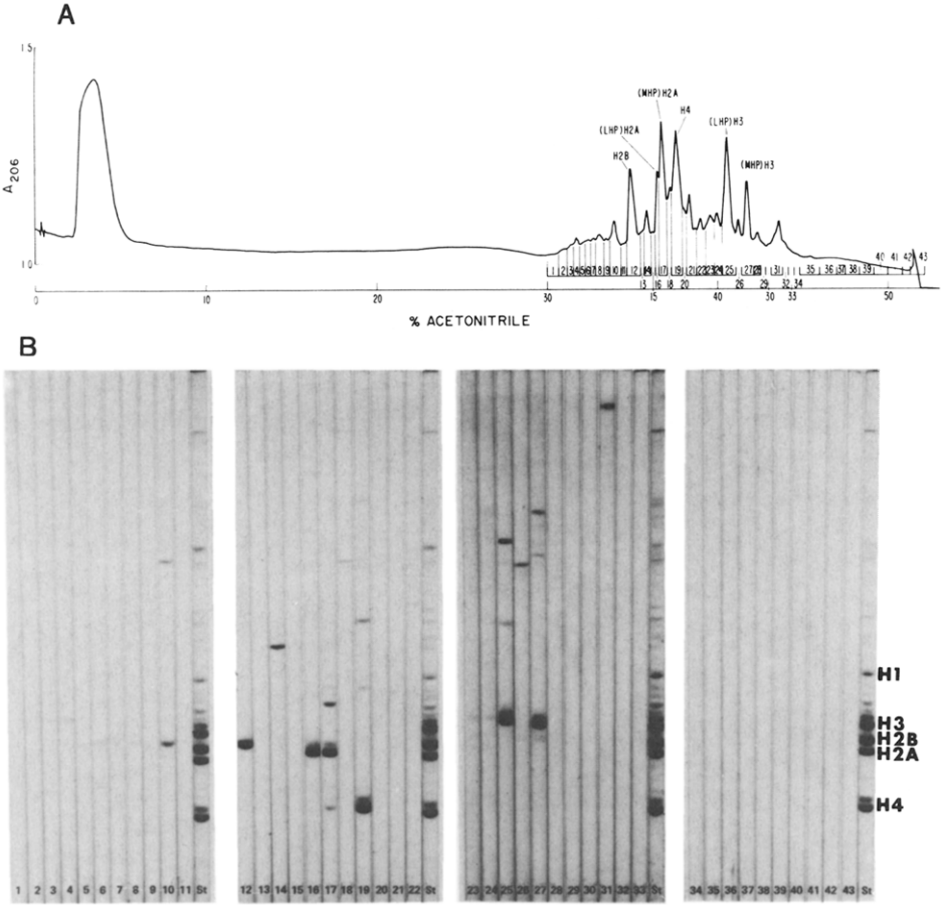

Fig. 4. Preparation and analysis of HPLC fractions from isolated CHO chromosomes. A, $1.6 \cdot 10^8$ chromosomes (equivalent to $2.6 \cdot 10^7$ nuclei), dissolved in 1000 μl 6 *M* Gu · HCl containing 0.2% TFA, were injected into a μBondapak CN column, equilibrated with 6 *M* Gu · HCl containing 0.2% TFA. The column was eluted as shown in Fig. 2. Effluent fractions were collected as indicated in this figure and lyophilized. B, The HPLC fractions indicated were subjected to acid–urea polyacrylamide gel electrophoresis along with whole histone preparations, which were used as identification standards (St).

it appears that peak D from nuclei (Fig. 3) is either missing or unresolved from H2B in chromosomes (Fig. 4). Other differences were also observed in Fig. 4, such as extra peaks in chromosomes between H2B and (LHP)H2A, between H4 and LHP(H3), between (LHP)H3 and MHP(H3), and between (MHP)H3 and fraction 31 (where LHP and MHP refer to less-hydrophobic and more-hydrophobic variants). At this point it was not known whether these extra peaks resulted from better resolution of proteins in chromosome preparations or from intrinsic differences in the proteins of nuclei and chromosomes.

In order to differentiate between these two possibilities, nuclei labeled with [^{14}C]lysine, and chromosomes labeled with [^{3}H]lysine were mixed and subjected to HPLC (Fig. 2C–E). (Note: the nuclei were isolated from interphase cells in the pres-

ence of propidium iodide by the same method as that used to isolate chromosomes from mitotic cells.) No significant differences were observed between the [^{3}H]protein elution pattern of the chromosomes (Fig. 2D) and the [^{14}C]protein elution pattern of the nuclei (Fig. 2E). Thus, it was concluded that when the same isolation procedures were used for nuclei and chromosomes, there were no major qualitative differences between nuclei and chromosomes when this HPLC method was used and that the increased number of peaks in chromosomes probably resulted from increased resolution due to smaller sample sizes. Propidium iodide did not appear to interfere with the HPLC system, with protein detection, or with protein retention times.

Chromatography of nuclei on Bio-Sil TSK gel filtration columns

From the experiments described above it was concluded that an additional chromatography of some kind was needed before the reversed-phase chromatography to separate the dissolved nuclei into DNA, guanidine and total protein. If this could be accomplished, DNA and guanidine could be diverted from the HPLC

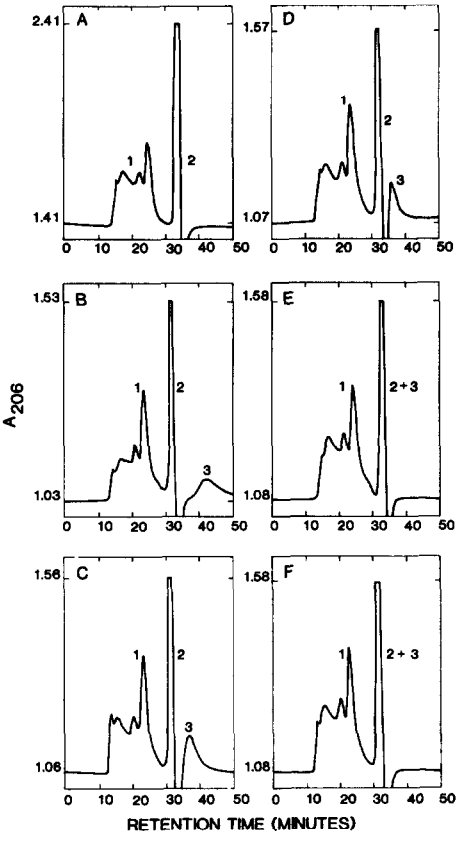

Fig. 5. Fractionation of dissolved nuclei by gel filtration chromatography on a Bio-Sil TSK guard column followed by a Bio-Sil TSK 400 column. The columns were equilibrated with the eluent and then injected with 4.5 · 10^6 nuclei dissolved in 15 μl 6 *M* Gu · HCl containing 0.2% TFA. The columns were eluted isocratically at 0.5 ml/min with aqueous solvents containing 0.2% TFA and (A) 0%, (B) 5%, (C) 10%, (D) 15%, (E) 20%, (F) 25% acetonitrile.

system, and the proteins could be loaded on the reversed-phase column for chromatography without loss of H1 or other weakly adsorbed proteins. In an attempt to accomplish this, dissolved nuclei were injected into an HPLC system consisting of a Bio-Sil TSK guard column, followed by a Bio-Sil TSK 400 gel filtration column, both of which were equilibrated with water containing 0.2% TFA. Two regions of the chromatogram were fractionated: (1) a complex of components following the exclusion limit, presumed to contain the proteins, and (2) a sharp peak at the inclusion limit, containing the guanidine (Fig. 5A). Since it was anticipated that this system would be used with a reversed-phase column which used acetonitrile as an eluent, the effects of acetonitrile on Bio-Sil TSK chromatography were examined. It was found that at 5–15% acetonitrile a third peak was eluted after the inclusion limit (Fig. 5B–D). This peak was eluted closer to the inclusion limit when the acetonitrile concentration was increased. At 20% and 25% acetonitrile, this peak was eluted in the inclusion limit with the guanidine (Fig. 5E and F). At 50% acetonitrile, it actually was eluted ahead of the guanidine peak (Fig. 6C). The elution times of the TSK fractions 1 and 2 were unaffected by acetonitrile concentration. Thus, it appears that

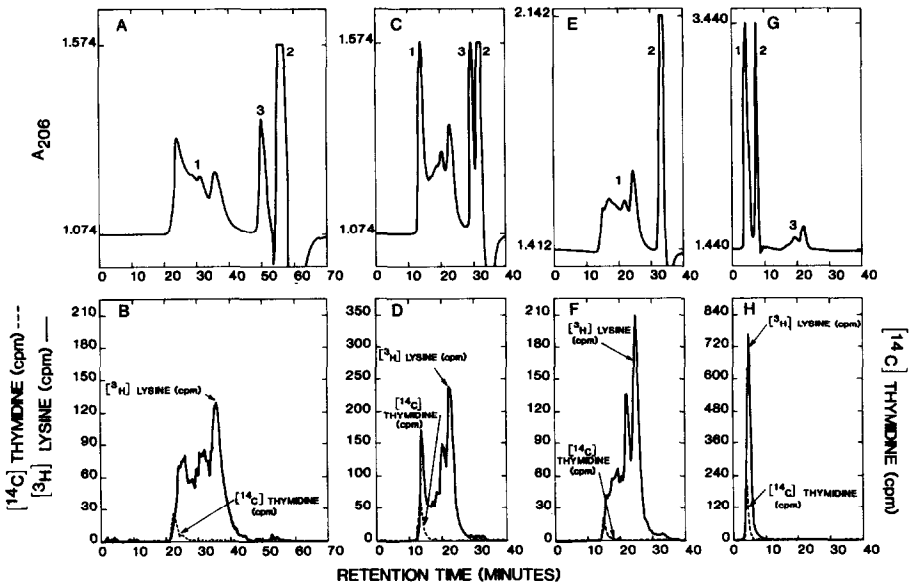

Fig. 6. Determination of DNA and protein in Bio-Sil TSK gel chromatography fractions of dissolved nuclei. A variety of combinations of Bio-Sil TSK columns were equilibrated with the eluents. Nuclei containing [^{14}C]thymidine labeled DNA and [^{3}H]lysine labeled protein were dissolved in 6 *M* Gu · HCl containing 0.2% TFA and loaded onto the HPLC system at a concentration of 2.8 · 10^6 nuclei/10 μl injection. The columns were eluted isocratically at 0.5 ml/min with either water containing 0.2% TFA or acetonitrile–water (50:50) containing 0.2% TFA as indicated below. Fractions of the effluent were collected every 0.5 min (0.25 ml per fraction) and their radioactivity was determined. A, B: TSK guard column followed by a TSK 400 column followed by a TSK 250 column eluted with water–acetonitrile (50:50) containing 0.2% TFA. C, D: TSK guard column followed by a TSK 400 column eluted with water–acetonitrile (50:50) containing 0.2% TFA. E, F: TSK guard column followed by a TSK 400 column eluted with water containing 0.2% TFA. G, H: TSK guard column (alone) eluted with water containing 0.2% TFA. B, D, F and H present the radioactivities of the fractions eluted in A, C, E and G, respectively. Peaks marked 1 contain protein; peak 2 contains guanidine; peak 3 contains the US fraction.

TSK fraction 3 has significant secondary interactions with this gel filtration packing while the proteins do not.

In order to determine where the DNA and proteins were eluted from the Bio-Sil TSK columns, nuclei containing DNA labeled with [^{14}C]thymidine, and protein labeled with [^{3}H]lysine, were dissolved in 6 *M* Gu · HCl containing 0.2% TFA and loaded onto a variety of TSK column combinations (Fig. 6). It was found that only a small amount of DNA was eluted at the exclusion limit at the beginning of the chromatography (Fig. 6B, D, F and H). Most of the DNA was irreversibly adsorbed on the TSK guard column (Fig. 6G and H). Most important, measurements of the ^{3}H-labeled protein loaded on, and eluted from, the column indicated that all the protein in the sample was eluted in the complex of peaks marked as 1 following the exclusion limit (Fig. 6B, D, F and H). No significant amount of ^{3}H-labeled protein was detected with the guanidine (peak 2) in the total inclusion peak, whether or not acetonitrile was present in the eluent. TSK fraction 3 also did not contain either protein or DNA.

Chromatography of nuclei on Bio-Sil TSK–μBondapak CN column combinations

Since the guard column alone was sufficient to accomplish the removal of most of the DNA from the sample without adsorbing any of the proteins (Fig. 6G and H), it was decided to use this column as a precolumn to remove DNA from samples being loaded onto the μBondapak CN reversed-phase chromatographic system. A large sample of nuclei (10 times larger than that in Fig. 6G), containing [^{14}C]DNA and [^{3}H]protein, was dissolved and loaded onto the Bio-Sil TSK guard column, which was connected directly to the inlet of a μBondapak CN column, both equilibrated with water containing 0.2% TFA. All three TSK fractions were eluted isocratically with water containing 0.2% TFA from the TSK guard column (Fig. 7A) onto the μBondapak CN column. The CN column was then eluted with an acetonitrile gradient containing 0.2% TFA by passing the solvent through both columns (Fig. 7D). The guanidine eluted from the CN column in the void volume contained the small amount of DNA (Fig. 7E) which passed through the TSK guard column (Fig. 7B). Only a small fraction of [^{3}H]protein was observed in the void volume (Fig. 7F). Histone H1 was eluted from the CN column along with the other histones and a large array of non-histone proteins (Fig. 7D and 7F). Thus, it appeared that the TSK column solved two of the major problems previously encountered, interference with chromatography by DNA and interference of protein adsorption by guanidine, which caused protein loss.

The only remaining problem with this HPLC system was the presence of the US fraction, which obscured analysis in the H1 region (Fig. 7D). Similarities in the size of the US peak (Fig. 7D) and the TSK fraction 3 (Fig. 7A), and the fact that neither of these peaks contained protein (Fig. 7C and 7F) suggested that they might be the same material. In order to determine whether this was true, the TSK guard column was removed from the HPLC system after TSK fractions 1 and 2 had passed through it but before TSK fraction 3 was eluted, thus preventing fraction 3 from being loaded onto the CN column. The CN column was then eluted with the acetonitrile gradient (Fig. 8A). The histones (including H1) and the non-histone proteins were eluted between 25–50% acetonitrile and the guanidine was eluted in the void volume. However, the US fraction was missing, indicating that TSK fraction 3 con-

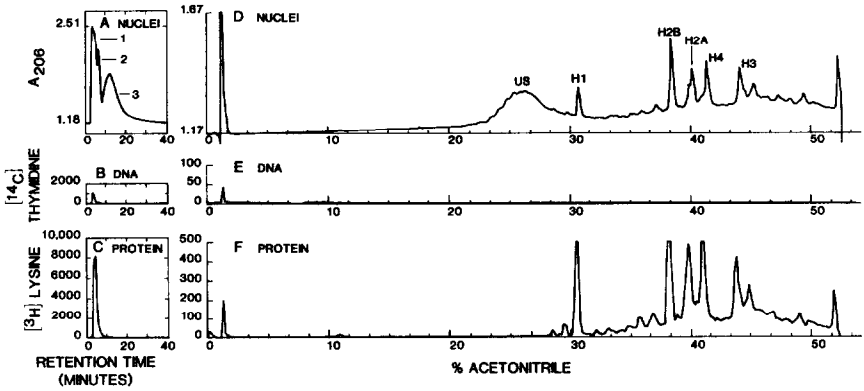

Fig. 7. HPLC of proteins from dissolved nuclei on a Bio-Sil TSK guard column followed by a μBondapak CN column. Nuclei containing [^{14}C]thymidine labeled DNA and [^{3}H]lysine labeled protein were dissolved in 6 *M* Gu · HCl containing 0.2% TFA. Ten times the amount used in Fig. 6G ($2.8 \cdot 10^7$ nuclei) in 100 μl was injected into the Bio-Sil TSK guard column. A, The TSK Guard column was eluted isocratically with water containing 0.2% TFA at 0.5 ml/min, producing three fractions (1–3). The effluent from the TSK guard column was collected in 0.5-min fractions (0.25 ml per fraction) and the radioactivity of [^{14}C]DNA (B), and the [^{3}H]protein (C) was determined. D, The nuclear material injected into the Bio-Sil TSK guard column was eluted directly into a μBondapak CN column with water containing 0.2% TFA at 1 ml/min. After 30 min (when all three TSK fractions had been eluted from the TSK column), the TSK–CN column combination was eluted at 1 ml/min with a 0–50% acetonitrile gradient containing 0.2% TFA, as described in Fig. 1. The effluent from the μBondapak CN column was collected in 1-ml fractions, and the radioactivity of the [^{14}C]DNA (E) and the [^{3}H]protein (F) was determined.

tained this material. This fact was confirmed by eluting fraction 3 from the TSK column into a CN column and then observing the elution of US from the CN column at 24–30% acetonitrile.

It was thought that the above procedure could be used to remove guanidine from the reversed-phase system also. However, the resolution between the TSK protein fraction (No. 1) and the TSK guanidine fraction (No. 2) was poor when large samples were injected (Fig. 7A), making it impossible to obtain protein loading on the CN column without some guanidine contamination. It had been observed that the addition of a Bio-Sil TSK 400 column after the TSK guard column produced good resolution between TSK fractions 1 and 2 (Fig. 6E). Thus, a TSK 400 column was inserted between the TSK guard column and the μBondapak CN column to generate enough separation between the protein and guanidine to facilitate the loading of only the TSK fraction 1 protein peaks onto the reversed-phase column. When this was done, a chromatogram containing all the histones (including H1) and non-histone proteins was obtained from the CN column without interference from DNA, guanidine, or the US fraction (Fig. 8B).

Chromatography of nuclei on Bio-Sil TSK–Zorbax C_8 column combinations

In order to determine whether reversed-phase columns other than μBondapak CN could be used for nuclei protein analysis, a Zorbax C_8 column was installed in the HPLC system following a Bio-Sil TSK guard column. When a sample of dissolved nuclei was eluted with an acetonitrile gradient containing 0.2% TFA, non-histone proteins appeared to be better resolved by the Zorbax C_8 column (Fig. 9A). The use of morpholine markedly improved the sharpness of the non-histone peaks (Fig. 9B).

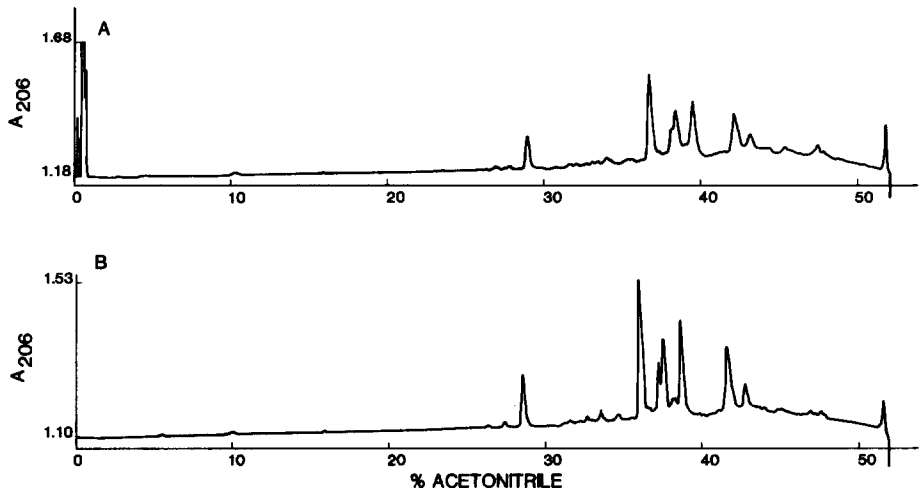

Fig. 8. Elimination of the US fraction and the guanidine fraction from the μBondapak CN column by manipulation of the Bio-Sil TSK column effluent. A, A Bio-Sil TSK guard column followed by a μBondapak CN column was loaded with dissolved nuclei as in Fig. 7. TSK fractions 1 and 2 (see Figs. 6G and 7A) were eluted from the TSK column into the CN column in 9 min at 0.5 ml/min with water containing 0.2% TFA. The TSK column was disconnected from the CN column and TSK fraction 3 was diverted to waste. The μBondapak CN column was then eluted with an acetonitrile gradient, containing 0.2% TFA at 1 ml/min, as described in Fig. 1. B, A Bio-Sil TSK guard column followed by a Bio-Sil TSK 400 column, followed by a μBondapak CN column, was loaded with dissolved nuclei as in Fig. 7. TSK fraction 1 (see Figs. 5A and 6E) was eluted from the TSK columns into the CN column in 30 min at 0.5 ml/min with water containing 0.2% TFA. The TSK columns were disconnected, and TSK fractions 2 and 3 were diverted to waste. The μBondapak CN column was then eluted with an acetonitrile gradient containing 0.2% TFA at 1 ml/min, as described in Fig. 1.

However, when morpholine was used with the μBondapak CN column no significant increase in peak sharpness was observed. This suggests some silanol activity in the Zorbax C_8 column. Several disadvantages were also observed: some of the H2A variants were not separated from H4, and the US fraction was eluted with histone H2B. This latter problem was solved by loading only TSK fractions 1 and 2 onto the Zorbax C_8 column (Fig. 9C). From these results it appears that the Zorbax C_8 column may offer some advantages for fractionating the non-histone proteins if one is not interested in fractionating the histones as completely as possible.

Chromatography of nuclei on Bio-Sil TSK–C_{18} reversed-phase columns

In our previous work on histone fractionation by HPLC we had found that the μBondapak C_{18} Radial-Pak column produced the best resolution of histones[3,4]. Therefore, we examined that column and the Nova-Pak C_{18} Radial-Pak column to determine whether they offered advantages for nuclear protein analysis. The Nova-Pak C_{18} column was chosen because it contains a spherical silica packing and could offer some of the advantages seen with the spherical packing of the Zorbax C_8 column. However, when dissolved nuclei were eluted from the Bio-Sil TSK guard–Nova-Pak C_{18} Radial-Pak column combination, the protein resolution was found to be very poor (Fig. 10A). The addition of morpholine to the solvents did not increase

protein resolution on this column (Fig. 10B) as it had on the Zorbax C_8 column (Fig. 9B).

When the μBondapak C_{18} Radial-Pak column was used with a Bio-Sil TSK guard column, the histones were fractionated as expected, with resolution of the H2A and H3 variants (Fig. 10C). Morpholine did not improve the resolution nor increase peak sharpness for either histones or non-histone proteins (Fig. 10D). It appears that this column remains the column of choice for histone variant fractionation.

The US fraction was eluted from both of the reversed-phase C_{18} columns between the H3 variants at acetonitrile concentrations greater than 50%. Comparing the various columns examined in this report, we found that the retention time of US on reversed-phase columns increases with the hydrophobicity of the column, *i.e.*, US retention time increases in the following order: CN < C_8 < C_{18}. The identity of this material remains to be established. At the present, we can only say that its interaction with modified silica HPLC packings is greatly dependent on both the polarity of the mobile phase solvent (Fig. 5) and the hydrophobicity of the stationary phase (Figs. 7, 9, and 10). Thus, this unidentified substance might be expected to be lipid or carbohydrate in nature, or perhaps it is one of the detergents used to isolate nuclei.

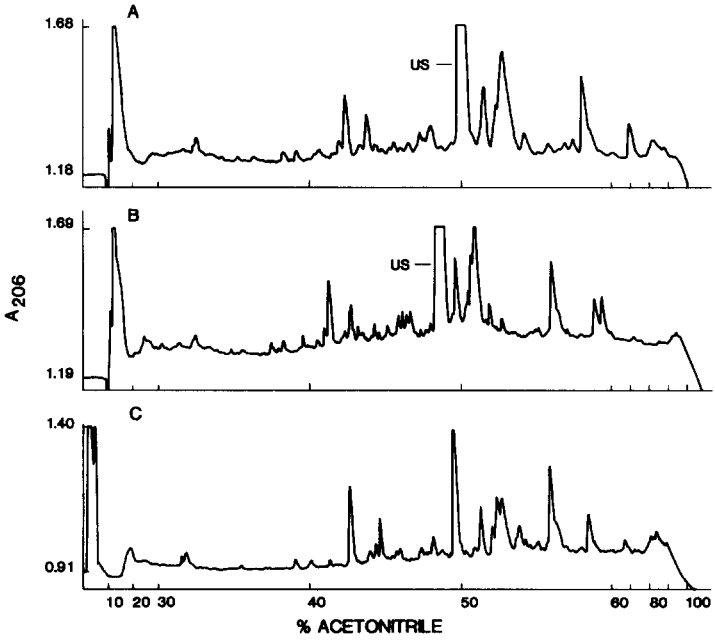

Fig. 9. Fractionation of dissolved nuclei with a Bio-Sil TSK guard column, followed by a Du Pont Zorbax C_8 column. Nuclei ($2.8 \cdot 10^7$) dissolved in 100 μl of 6 *M* Gu · HCl containing 0.2% TFA were injected into the columns, which were equilibrated with water containing 0.2% TFA. Proteins were then eluted by passing the following linear acetonitrile gradients containing 0.2% TFA through both columns at 0.5 ml/min: 0–30% acetonitrile in 30 min, 30–60% acetonitrile in 180 min, 60–100% acetonitrile in 30 min. A, Eluents contained no morpholine. B, Eluents contained 0.1% morpholine. C, TSK fractions 1 and 2 were eluted from the Bio-Sil TSK guard column into the Zorbax C_8 column with a 9-min isocratic flow of water containing 0.2% TFA and 0.1% morpholine at 0.5 ml/min. The TSK guard column was removed, and TSK fraction 3 was diverted to waste. The Zorbax C_8 column was then eluted with the three linear acetonitrile gradients, as described above, using eluents containing 0.1% morpholine.

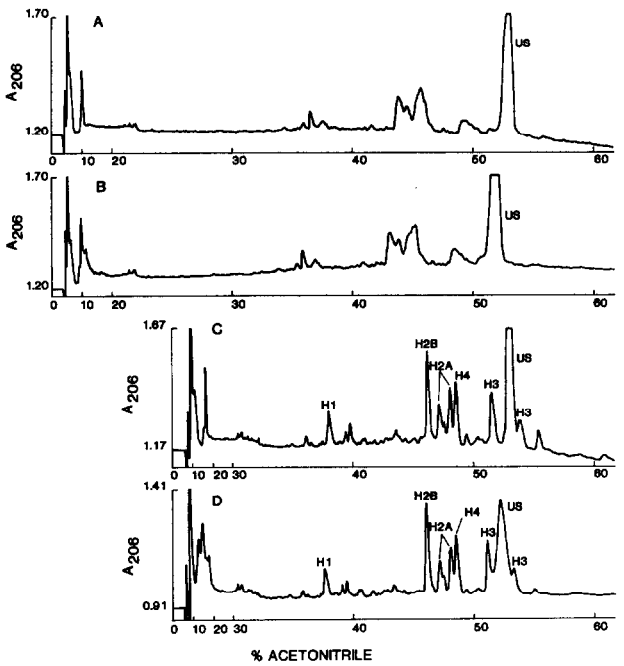

Fig. 10. Fractionation of dissolved nuclei with a Bio-Sil TSK guard column, followed by either a Waters Nova-Pak C_{18} Radial-Pak column or a Waters μBondapak C_{18} Radial-Pak column. The columns were equilibrated with the initial elution solvent and injected with $3 \cdot 10^7$ nuclei dissolved in 100 μl 6 *M* Gu · HCl containing 0.2% TFA. The columns were eluted by passing eluents containing 0.2% TFA through both columns at 1 ml/min. A, Bio-Sil TSK guard column, followed by a Nova-Pak C_{18} Radial-Pak column, eluted with a 0–20% acetonitrile linear gradient in 30 min, followed by a 20–60% acetonitrile linear gradient in 240 min. No morpholine was added to these eluents. B, Same as the TSK guard–Nova-Pak C_{18} experiment above, except that all eluents contained 0.1% morpholine. C, Bio-Sil TSK guard column, followed by a μBondapak C_{18} Radial-Pak column, eluted with a 0–30% acetonitrile linear gradient in 30 min, followed by a 30–60% acetonitrile linear gradient in 180 min. No morpholine was added to these eluents. D, Same as the TSK guard–μBondapak C_{18} experiment above, except that all eluents contained 0.1% morpholine.

DISCUSSION

The idea that it might be possible to load a preparation of whole nuclei or whole chromosomes directly onto an HPLC system and obtain a fractionation, analysis, and preparation of nucleoproteins in one step is attractive to the life scientist interested in nuclear function. In this report we have described experiments which led to the successful development of such a method. In our initial experiments we used the procedure of Henderson *et al.*[5] for structural dissociation by guanidine hydrochloride to dissolve whole nuclei or chromosomes. This solution was then subjected to our reversed-phase HPLC procedure for histones[1–4]. However, chromatography was vitiated by two major problems: (1) column fouling by the large amounts of DNA in the samples of mammalian cell nuclei, and (2) the presence of a large amount of an unidentified non-protein substance (US) which obscured the H1 histone and some non-histone proteins in the chromatogram.

These problems were solved by installing Bio-Sil TSK gel filtration columns ahead of the reversed-phase column. When dissolved nuclei were passed through a Bio-Sil TSK guard column, the DNA was irreversibly adsorbed on it. The remainder of the sample was passed through this column into a Bio-Sil TSK 400 column. Proteins were eluted from this column between the exclusion and inclusion limits, followed by the guanidine at the inclusion limit. Fortunately, the non-protein US fraction exhibited a significant interaction with the TSK columns and was not eluted from them until after the guanidine. Thus, by simple effluent stream manipulation it was possible to direct the proteins from the TSK columns to the reversed-phase column and then to divert the guanidine and US fraction to waste. After several runs, the DNA-loaded TSK guard column packing was either discarded or purged of DNA by washing it with 6 *M* Gu · HCl containing 0.2% TFA.

This report present details of the properties of our chromatographic system which should be useful to researchers wishing to adapt it to specific experimental problems. Of primary importance are observations concerning DNA adsorption to rigid silica packings containing bonded hydrocarbon surfaces. The DNA was adsorbed to both TSK and CN columns, but appeared to be less tightly bound to the hydrophobic bonded phase of the CN column than it was to the hydrophilic bonded phase of the TSK columns. This suggests that while hydrophobic and hydrophilic forces are both important in removing DNA from the sample, the hydrophilic forces are stronger. As a result of the weaker hydrophobic forces between DNA and the μBondapak CN column, some DNA would be eluted from the μBondapak column during reversed-phase chromatography and, thus, interfere with chromatography and protein peak detection. By placing the more adsorbent TSK columns ahead of the μBondapak column, less than 5% of the loaded DNA ever reached the μBondapak column. That which did pass through the TSK column appeared to have no attraction for the μBondapak CN packing and was eluted from it in the void volume, thus causing no further problems.

On the other hand, the proteins appeared to exhibit no significant interaction with the hydrophilic bonded phase of Bio-Sil TSK in the presence of TFA. Even decreasing the polarity of the mobile phase solvent with 50% acetonitrile did not change the elution pattern of the proteins from the TSK columns. This is important for researchers wishing to use some acetonitrile in the initial eluents for reversed-phase HPLC in this system.

In contrast to both DNA and protein, the US fraction shows significant adsorption on both TSK and μBondapak columns. While its attraction to TSK is weaker than that of DNA, it is sufficient to separate this substance from the dissolved nuclei mixture, thus facilitating its elimination from the reversed-phase system and its recovery for identification studies.

This system was found to be applicable to both isolated nuclei and isolated chromosomes. At the stage of method development when this was demonstrated, no significant differences were observed between the nucleoproteins of these two preparations. However, this observation was made early in the course of our experiments when some proteins were still being lost from the system. Thus, the comparison of nuclei and chromosomes should be repeated with the TSK guard column to remove DNA. Under those conditions, and perhaps with a different reversed-phase column, one might be able to resolve different proteins in these two preparations.

Comparisons of several reversed-phase columns suggested that the Zorbax C_8 column might offer some advantages for the resolution of non-histone proteins in nuclei. On the other hand, the μBondapak C_{18} Radial-Pak column definitely gave superior resolution of histone variants. Thus, further method development work will continue in an attempt to find conditions which will resolve both classes of proteins equally well.

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