unconstrained analysis is used, then large negative coefficients or large deviations from 1.0 for the sum of all secondary structures will indicate that the basis set has been overextended. Adding contraints only hides to fact that the analysis has failed.

Acknowledgments

We thank Professor R. R. Becker for the use of his Beckman 120B amino acid analyzer.

References

Baker, C. C., & Isenberg, I. (1976) *Biochemistry 15*, 629–634.
Brahms, S., & Brahms, J. (1980) *J. Mol. Biol. 138*, 149–178.
Bree, A., & Lyons, L. E. (1956) *J. Chem. Soc.*, 2658–2670.
Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.
Chen, Y.-H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.
Chou, P. Y., & Fasman, G. D. (1977) *J. Mol. Biol. 115*, 135–175.
Feldman, R. J. (1976) *Atlas of Macromolecular Structure on Microfiche*, Tracor Jitco, Inc., Rockville, MD.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Greenfield, N., Davidson, B., & Fasman, G. D. (1967) *Biochemistry 6*, 1630.
Harman, H. H. (1976) *Modern Factor Analysis*, 3rd ed., The University of Chicago Press, Chicago.
Johnson, W. C., Jr. (1971) *Rev. Sci. Instrum. 42*, 1283–1286.
Johnson, W. C., Jr., & Tinoco, I., Jr. (1972) *J. Am. Chem. Soc. 94*, 4389–4390.
Lloyd, D. (1969) Ph.D. Thesis, University of California, Berkeley.
McMullen, D. W., Jaskunas, S. R., & Tinoco, I., Jr. (1967) *Biopolymers 5*, 589–613.
Moore, S. (1968) *J. Biol. Chem. 243*, 6281–6283.
Moore, S., & Stein, W. H. (1948) *J. Biol. Chem. 176*, 367–388.
Moore, S., & Stein, W. H. (1954) *J. Biol. Chem. 211*, 907–913.
Saxena, V. P., & Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 969–972.
Siegel, J. B., Steinmetz, W. E., & Long, G. L. (1980) *Anal. Biochem. 104*, 160–167.
Woody, R. W. (1969) *Biopolymers 8*, 669–683.

Nonhistone Proteins Cross-Linked by Disulfide Bonds to Histone H3 in Nuclei from Friend Erythroleukemia Cells†

Alice E. Grebanier‡ and A. Oscar Pogo*

ABSTRACT: Nonhistone proteins in close proximity to histone H3 were detected in cross-linking experiments. Disulfide bonds were formed in nuclei between histone H3 and nonhistone proteins containing sulfhydryl groups by oxidation with H_2O_2. The histones were solubilized either as nucleosomes extracted by 1 mM EDTA from micrococcal nuclease treated nuclei or else as DNA-free proteins extracted by a buffer containing 1 M KCl from nuclei that had been extensively digested with DNAase I. The extracts were examined for disulfide cross-linked proteins by two-dimensional electrophoresis. The nonhistone proteins appeared to be extracted selectively by the two extraction procedures; neither procedure was completely efficient in releasing the histones from the nuclei. One cross-linked nonhistone protein (M_r 46 000) was extracted in 1 mM EDTA along with the nucleosomes. The high salt buffer extracted many nonhistone proteins, including four cross-linked nonhistones which were not found in the EDTA extracts; three of the cross-linked proteins were about M_r 50 000 and one was about M_r 36 000. Four of the five cross-linked, nonhistone proteins which are extracted either by EDTA or high salt are found cross-linked to histone H3 in nuclei prepared from cells lysed in the presence of potassium iodoacetate. Only the disulfide bond to the M_r 36 000 protein requires highly oxidizing conditions for its formation. Thus, many of the disulfide bonds formed between H3 and nonhistone proteins may occur naturally. These experiments disclose a set of nonhistone proteins which are close enough to histone H3 that a disulfide bond can be formed between the histone and nonhistone. The close association implies that these proteins may have significant structural or functional roles in the nucleus.

Previous work from this laboratory has been concerned with preparation and characterization of the nuclear matrix (Miller et al., 1978), an ordered, skeletal structure within the nucleus (Berezney & Coffey, 1974). In the course of experiments with matrix prepared from nuclei of Friend erythroleukemia cells, the discovery was made that the morphology of the nucleus is preserved after almost all of the DNA has been removed by DNAase I digestion (Long et al., 1979). The intranucleosomal histones remain with the nuclear matrix after digestion of the DNA, and the interactions between the histones, as monitored by the use of cross-linking reagents, appear to be undisturbed (Long et al., 1979; Grebanier & Pogo, 1979). The histones are known to be organized into octameric units, the nucleosome cores, around which the DNA is wrapped (Kornberg, 1974; van Holde et al., 1974). However, the histones normally do not maintain such large structures at physiological ionic strength in the absence of DNA. Therefore, the interactions between the histones and the other

† From the L.F. Kimball Research Institute of the New York Blood Center, New York, New York 10021. *Received August 18, 1980.* This work was supported by grants from the National Institutes of Health and the National Science Foundation.

‡ Present address: 35–18 21 Avenue, Astoria, NY 11105.

0006-2960/81/0420-1094$01.25/0

nuclear proteins must be responsible for the preservation of nuclear morphology following DNAase I treatment. Identification of the nonhistone proteins involved and elucidation of the nature of their interactions with the histones are important subjects for further investigation.

Cross-linking experiments revealed a set of nonhistone proteins that seemed to be able to form disulfide bonds with histone H3 when the nuclei were oxidized with H_2O_2 (Grebanier & Pogo, 1979). These proteins are believed to be cross-linked to H3 because their off-diagonal positions on the two-dimensional electrophoretograms were appropriate for polypeptides which had been cross-linked to a protein the size of H3. Further, a streak of H3 running horizontally across the gel indicated the involvement of H3 in many kinds of cross-linking events. If these nonhistone proteins are in close and regular association with H3, they would be of particular interest in light of their possible role in forming a link between the histones and the nuclear matrix.

The present study was undertaken with the use of undifferentiated and differentiated Friend erythroleukemia cells (Friend et al., 1971) to explore the nature of the association of the cross-linked, nonhistone proteins with histone H3 and to find conditions under which the cross-linked complexes can be solubilized. To this end, disulfide bonds were formed between proteins in nuclei by oxidation with H_2O_2 and the histones extracted either as nucleosomes from micrococcal nuclease treated samples or as DNA-free proteins from DNAase I treated samples. The extracts were examined for cross-linked proteins by two-dimensional gel electrophoresis (Wang & Richards, 1974). Particular cross-linked, nonhistone proteins were extracted together with the histones by these two procedures.

Materials and Methods

Cell Culture. Friend murine erythroleukemia cells, line 745 (a gift from C. Friend), were grown in suspension cultures in Dulbecco's minimal essential medium as described previously (Long et al., 1979).

Preparation of Nuclei: H_2O_2 Treatment. Cells were washed and lysed as described previously (Long et al., 1979), except that Triton X-100 and Saponin were at the concentration of 0.375% (w/v) each in the cell lysis buffer. Nuclei were collected by centrifugation for 10 min at 250*g* and washed once with a buffer containing 100 mM KCl, 10 mM Tris[1]-HCl (pH 7.7 at 23 °C), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM PMSF, and 146 mM sucrose. The nuclei [(5×10^7)/mL] were treated with 20 mM H_2O_2 in the same buffer for 30 min in an ice bath, then collected by centrifugation and washed once more with the same buffer containing 25 mM potassium iodoacetate as an addition.

In some experiments, 25 mM potassium iodoacetate was added to the cell lysis buffer and the first wash buffer. The H_2O_2 treatment step was omitted.

Micrococcal Nuclease Digestion and EDTA Extraction. Nuclei [(5×10^7)/mL] in 100 mM KCl, 10 mM Tris-HCl (pH 7.7 at 23 °C), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM PMSF, and 146 mM sucrose were treated with 200 units/mL micrococcal nuclease (Worthington) for 45 min at 10 °C. The reaction was stopped by the addition of 0.25 volume of 100 mM EDTA (pH 7.0). The suspension was centrifuged for 10 min at 500*g*. The pellet was resuspended with 1 mM EDTA–0.5 mM PMSF (pH 7.0) to a concentration of 5×10^7 nuclei/mL. The suspension stood for 30 min in an ice bath and was then centrifuged for 10 min at 500*g*. The supernatant was saved.

DNAase I Digestion and High Salt Extraction. Nuclei were digested with DNAase I as described previously (Long et al., 1979). Following digestion, the suspension was layered over 5 volumes of 300 mM KCl, 25 mM potassium iodoacetate, 10 mM Tris-HCl (pH 7.7 at 23 °C), 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 146 mM sucrose, and the nuclei were collected by centrifugation for 10 min at 500*g*. The nuclei were resuspended to 4×10^8 nuclei/mL with 1 M KCl, 25 mM potassium iodoacetate, 10 mM Tris-HCl (pH 7.7 at 23 °C), 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 146 mM sucrose. The suspension stood for 30 min in an ice bath and was then centrifuged for 10 min at 500*g*. The supernatant was saved.

Sucrose Density Gradient Centrifugation. EDTA extract (0.5 mL) was layered on a 12-mL linear gradient [5–20% sucrose (w/v) in 10 mM Tris-HCl (pH 7.5 at 23 °C) and 1 mM EDTA]. Centrifugation was at 4 °C for 18 h at 30 000 rpm in an SW41 rotor (Beckman).

The high salt extract (0.5 mL) was layered on a 12-mL linear gradient [5 to 20% sucrose (w/v) in 1 M KCl, 10 mM Tris-HCl (pH 7.5 at 23 °C), 1.5 mM $MgCl_2$, and 0.5 mM PMSF]. Centrifugation was at 4 °C for 24 h at 35 000 rpm in an SW41 rotor (Beckman).

Gradients were analyzed with an ISCO gradient fractionator and an ultraviolet absorption recorder.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. EDTA or high salt extracts were concentrated in an Amicon ultrafiltration cell (PM30) filter. High salt extracts were diluted with H_2O and reconcentrated several times to reduce the amount of KCl in the sample. $NaDodSO_4$ was added to a final concentration of 2% (w/v), glycerol to 10% (v/v), and Tris-HCl (pH 6.8) to 50 mM. The samples were heated at 90 °C for 2 min. Two-dimensional gels were run as described previously (Grebanier & Pogo, 1979). The samples were reduced with 2-mercaptoethanol between the first and second dimensions.

Sucrose density gradient fractions were diluted so that KCl was present at 100 mM. The diluted samples were collected on 6 mm diameter GFC filters (Whatman) by vacuum filtration in a procedure derived from that of Thomas et al. (1979). The proteins were quantitatively adsorbed onto the filters. The filters were placed in the wells of a $NaDodSO_4$–polyacrylamide gel [3% stacking gel, 12.5% running gel, prepared according to Laemmli (1970)]. Sample buffer (Laemmli, 1970) was added to each well. The proteins eluted from the filters during electrophoresis.

Results

EDTA Extracts of Micrococcal Nuclease Treated Nuclei. Nuclei isolated from undifferentiated or differentiated Friend cells were oxidized with H_2O_2 and then digested with micrococcal nuclease at 10 °C for 45 min as described under Materials and Methods. In the particular experiments shown here, 6% of the DNA was rendered acid soluble by this digestion in the nuclei from undifferentiated cells and 10% in those from differentiated cells. When the material released from the nuclei during the digestion period was examined by two-dimensional electrophoresis, no cross-linked proteins were detected (data not shown). The nuclei were subsequently extracted with 1 mM EDTA to release the bulk of the nucleosomes. These extracts contain mainly oligonucleosomes, as shown by sucrose density gradient centrifugation (Figure 1). When the EDTA extracts were analyzed for cross-linked proteins, the histone H3 homodimer was the most abundant

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

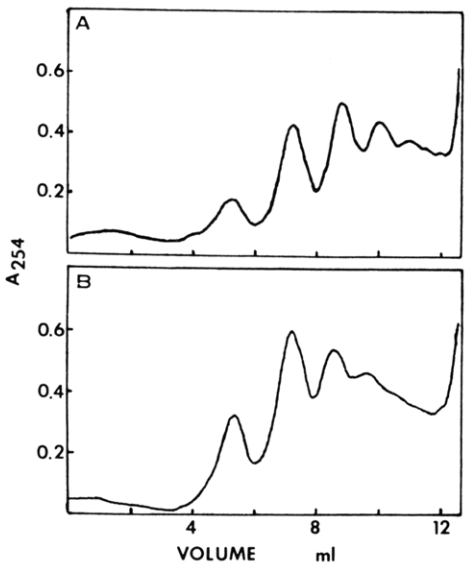

FIGURE 1: Sucrose density gradient centrifugation of EDTA extracts. (A) Profile of extract from 5×10^7 nuclei from undifferentiated Friend cells. (B) Profile of extract from 5×10^7 nuclei from differentiated Friend cells. Extraction and centrifugation procedures are described under Materials and Methods.

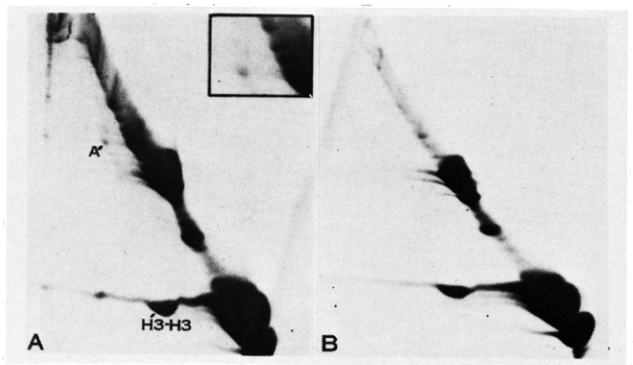

FIGURE 2: Two-dimensional electrophoresis of EDTA extracts. (A) Extract from 8×10^8 nuclei from undifferentiated Friend cells. (B) Extract from 8×10^8 nuclei from differentiated Friend cells. Extracts were concentrated and electrophoresis was performed as described under Materials and Methods. Inset is larger print of area of electrophoretogram with off-diagonal spots.

species detected (Figure 2). In samples extracted from nuclei of undifferentiated cells, the most prominent nonhistone protein shown to be involved in cross-linking was a protein of M_r ~46 000 (A in Figure 2A). The off-diagonal spot of protein A is vertically aligned with an off-diagonal spot of histone H3, and the mobility on the first dimension of the oligomer from which these spots are derived is appropriate for a protein with the size of a complex of protein A and histone H3 (Grebanier & Pogo, 1979). Thus, the formation of a disulfide bond between these two proteins is proven.

In other experiments not shown here, the nuclei from undifferentiated cells were treated with micrococcal nuclease at 37 °C for 10 min; digestion proceeded until 25–50% of the DNA was acid soluble. In these experiments, the EDTA extract consisted mainly of mononucleosomes, and the amount of off-diagonal protein A on the two-dimensional electrophoretograms was somewhat diminished. $NaDodSO_4$–polyacrylamide gel electrophoresis of fractions taken after sucrose density gradient centrifugation of the EDTA extract showed protein A in the fractions corresponding to the mononucleosome peak.

In contrast to the results with nuclei from undifferentiated cells, cross-linked, nonhistone proteins were not seen in EDTA

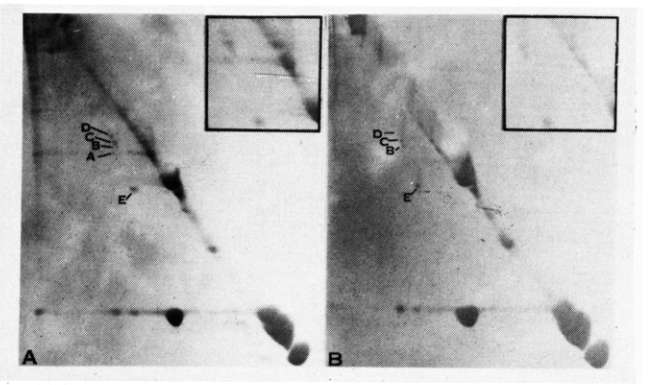

FIGURE 3: Two-dimensional electrophoresis of high salt extracts. (A) Extract from 2×10^8 nuclei from undifferentiated Friend cells. (B) Extract from 2×10^8 nuclei from differentiated Friend cells. Extracts were concentrated and electrophoresis was performed as described under Material and Methods. Insets are larger prints of area of electrophoretogram with off-diagonal spots.

extracts of micrococcal nuclease digested nuclei from differentiated cells (Figure 2B). Earlier work (Grebanier & Pogo, 1979) showed that very little of protein A is cross-linked and that the level of disulfide bond cross-linking is generally lower in nuclei from differentiated cells.

Thus, most of the nonhistone, cross-linked proteins which are seen on two-dimensional electrophoretograms of whole nuclei are not found to be associated with isolated nucleosomes. Protein A is the only nonhistone protein whose association with histone H3 is definitively shown by the experiments just described. However, the cross-linked, nonhistone proteins that are not extracted may also be associated with histone H3. In the experiments described above, the nuclear structures remaining after EDTA extraction contained 20% (nuclei from undifferentiated cells) and 15% (nuclei from differentiated cells) of the original DNA. $NaDodSO_4$–polyacrylamide gel electrophoresis of the proteins in the residual structures showed that substantial quantities of the histones were not extracted by the hypotonic EDTA solution (data not shown). The nonhistone proteins may be responsible, in part, for the retention of a large fraction of the nucleosomes after EDTA extraction.

High Salt Extracts of DNAase I Treated Nuclei. Following extensive digestion of nuclear DNA with DNAase I, the intranucleosomal histones are extracted coordinately into a buffer containing 1 M KCl (Long et al., 1979). As described under Materials and Methods, high salt extracts were obtained from H_2O_2-treated, DNAase I digested nuclei and analyzed by two-dimensional electrophoresis.

The most obvious cross-linked, nonhistone proteins in the high salt extract of nuclei from undifferentiated cells were three proteins of M_r ~50 000 (B, C, D in Figure 3A) and a protein of M_r ~36 000 (E in Figure 3A). Protein A was only marginally detectable in the off-diagonal region of the two-dimensional electrophoretogram of the extract and somewhat more evident in the high molecular weight aggregate which formed a line down the left side of the gel in the second dimension.

High salt extracts of nuclei from differentiated cells were similar to those from undifferentiated cells, except that protein A was not detectable on the two-dimensional electrophoretograms (Figure 3B).

The salt-extracted proteins were separated on sucrose density gradients. The ultraviolet absorption profile showed two major, overlapping peaks (Figure 4A). The slower peak contained mainly H2A and H2B, while the faster contained mainly H3 and H4 (Figure 4B). Proteins A, B, C, D, and E were found

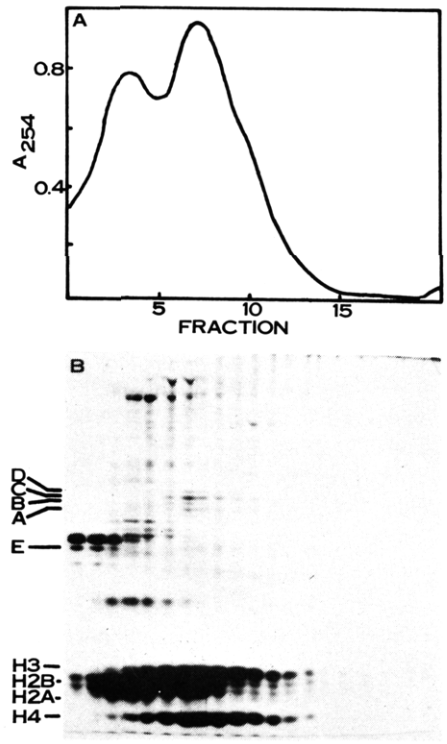

FIGURE 4: High salt extract of nuclei from undifferentiated Friend cells. (A) Sucrose density gradient centrifugation profile of high salt extract from 2×10^8 nuclei. Extraction and centrifugation procedures are described under Materials and Methods. Sedimentation is from left to right. (B) $NaDodSO_4$–polyacrylamide gel electrophoresis of proteins in fractions taken from sucrose density gradients in (A). One-third of each fraction was prepared for electrophoresis as described under Materials and Methods.

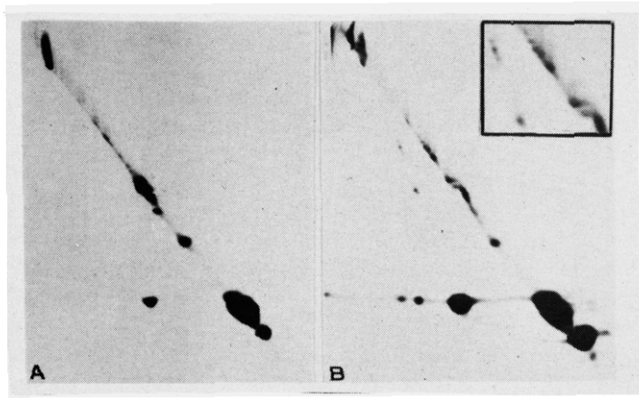

FIGURE 5: Two-dimensional electrophoresis of proteins in slow and fast peaks. (A) Slow peak. (B) Fast peak. High salt extracts were subjected to sucrose density gradient centrifugation as in Figure 4. The two peaks were collected separately, and material from six gradients was combined. The samples were dialyzed against H_2O and concentrated in an Amicon ultrafiltration cell. Electrophoresis was performed as described under Materials and Methods. Inset is larger print of area of electrophoretogram with off-diagonal spots.

principally in the region of the faster peak. The two peaks were collected separately, concentrated, and analyzed by two-dimensional electrophoresis (Figure 5). The nonhistone proteins were detected lying under the diagonal only in the electrophoretogram of the faster peak (Figure 5B). One off-diagonal spot of H3 was vertically aligned with protein E, and a second off-diagonal spot of H3 fell under the group of the other nonhistones. This result indicates that all of these proteins had been cross-linked to histone H3.

Sucrose density gradient centrifugation and two-dimensional electrophoresis of extracts from differentiated cells gave similar results (data not shown).

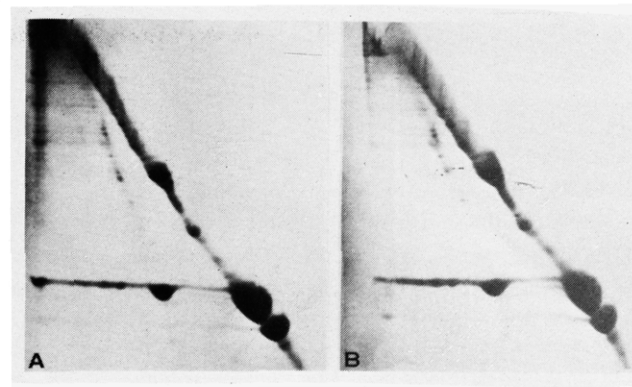

FIGURE 6: Two-dimensional electrophoresis of proteins in nuclei treated with H_2O_2 or with potassium iodoacetate. (A) 10^8 nuclei treated with H_2O_2. (B) 10^8 nuclei from cells lysed in the pressure of potassium iodoacetate. Samples were prepared and electrophoresis was performed as described under Materials and Methods.

As was true for EDTA extraction of micrococcal nuclease treated nuclei, high salt extraction of DNAase I treated nuclei was not completely efficient in removing the histones from the fundamental structure.

The residual nuclear structures left after micrococcal nuclease digestion and EDTA extraction could be further digested with DNAase I and extracted with high salt. Even then, some histones were left with a residual structure. Two-dimensional electrophoretograms of these high salt extracts were similar to those from nuclei which were digested only with DNAase I. The second DNA digestion step was necessary to release the cross-linked, nonhistone proteins from micrococcal nuclease treated samples; high salt extraction immediately following the EDTA extraction did not release these proteins, even if the KCl concentration was raised to 2 M.

Possible Natural Occurrence of Disulfide Bonds between Nonhistone Proteins and Histone H3. The group of proteins which appear to be able to cross-link with H3 through the formation of disulfide bonds was first detected in samples of nuclei which had been oxidized with H_2O_2 (Grebanier & Pogo, 1979). The cross-links formed quite readily in samples which had been previously reduced by incubation with dithiothreitol; cross-linking would develop if the samples were left standing in the cold, even if H_2O_2 was not added. To test the possible occurrence of these disulfide bonds within the living cells, we added iodoacetate to the resuspension buffer just before the cells were lysed with detergent to release the nuclei. The iodoacetate should react with any free sulfhydryl groups exposed during the lytic process. When a sample that had been lysed in the presence of iodoacetate was compared to a sample that had been oxidized with H_2O_2, many of the same proteins were detected lying under the diagonal line across the two-dimensional electrophoretograms (compare A and B in Figure 6). The notable exception was protein E, which was not seen in the samples which were blocked with iodoacetate. Protein E was not found on the two-dimensional electrophoretograms of high salt extracts from these samples; in electrophoretic analyses of fractions taken from sucrose density gradients after centrifugation of the high salt extracts, protein E was not found in any portion of the gradient (data not shown). Thus, protein E is not extracted from DNA-depleted nuclei by high salt except when it is linked to H3 by a disulfide bond which is formed only under highly oxidizing conditions.

Although the possibility of oxidation of the sulfhydryl groups during lysis cannot be absolutely ruled out in these experiments, one may conclude that many of the disulfide bonds detected by two-dimensional electrophoresis can form in the

nucleus while it is still in the cell.

Discussion

The nonhistone proteins that are near the histones in the nucleus are of interest because of their possible role in regulation of genetic function. Workers in several laboratories have looked for nonhistone proteins associated with isolated nucleosomes and have found particular nonhistone proteins in certain subgroups of nucleosomes (Bakayev et al., 1977, 1978). The claim has been made that some of the high mobility group proteins (Goodwin et al., 1973) are preferentially associated with nucleosomes in transcriptionally active chromatin (Vidali et al., 1977; Mathew et al., 1979; Levy-W. et al., 1979).

In using cross-linking, we have taken a different approach to finding nonhistone proteins which are in close proximity to the histone, an approach which avoids the problem of possible rearrangement of proteins during preparation of samples. This problem is inherent in nucleosome isolation experiments: adventitious binding of some small high mobility group proteins to isolated nucleosomes has been found (Jackson et al., 1979). In our experiments, disulfide bonds are formed between proteins as an early step in the preparation of nuclei, before much opportunity arises for rearrangement of elements. Indeed, many of the disulfide bonds may exist before the nuclei are released from the Friend erythroleukemia cells. Then, using two different extraction procedures in which the bulk of the protein released is histone, we look for cross-linked proteins. Thus, we are using the sulfhydryl group of histone H3 as a "hook" to pull out a particular set of nonhistone proteins that interact with the histone.

One difficulty with our approach is that we do not have the means to quantitate the relative proportions of proteins on the two-dimensional electrophoretograms. The staining intensity of the off-diagonal spots of histone H3 appear to the eye to be much heavier than the spots of nonhistone proteins with which they are vertically aligned. However, since the spots of histone H3 appear on electrophoretograms only in conjunction with the spots of nonhistone proteins, there is no need to postulate the existence of other complexes of histone H3, either with itself or with other, unstained elements. Most probably, the nonhistones are less effectively stained with Coomassie blue than the histone is. Also, we have been unable to determine the proportion of the histone which is cross-linked to the nonhistone proteins. The gels are heavily overloaded for the histones so that the cross-linked proteins can be detected. Clearly, only a small fraction of H3 is cross-linked, since the bulk of H3 is found on the diagonal of the electrophoretograms; proteins on the diagonal have not been cross-linked. Though the fraction of the histone in contact with these nonhistone proteins is small, the association of these elements must be regularly repeated in order for the cross-linking to be detected at all. The cross-linking can be taken as evidence for specialized structures found at particular locations.

Another difficulty is that neither of our procedures extracts all of the histone. Consequently, we cannot draw any conclusions about the cross-linked, nonhistone proteins that are not extracted. Since we see no evidence of differential extraction between histones H2A, H2B, H3, and H4, we guess that nucleosomes may differ in terms of the elements that anchor them to the underlying nuclear structure.

The profile of the released proteins depends upon the protocol followed to prepare the extract. In extracts that contain nucleosomes, i.e., both the DNA and histones, the major cross-linked, nonhistone protein found is protein A. By contrast, cross-linked protein A is only marginally detectable in the off-diagonal region of a two-dimensional electrophoretogram of a high salt extract of DNAase I treated nuclei; the four prominent cross-linked proteins are B, C, D, and E.

The differences in the pattern of released nonhistone proteins may be caused by the involvement of different kinds of bonds to stabilize the normal interactions between proteins in the nucleus. During the isolation of nucleosomes, the release of the histones results from conformational changes in the DNA caused by the lower ionic strength; in nuclei which have been extensively digested with DNAase I, the histones are not released in hypotonic solutions. When the DNA is only partially digested, as with micrococcal nuclease, the tight association between the DNA and the histones results in the release of most of the histones from the nuclear structures in hypotonic EDTA. Relatively little nonhistone protein is released though. In the extensively digested nuclei, solubilization of proteins by high salt is the result of disruption of electrostatic interactions between polypeptides. Many nonhistone proteins are released, as well as a large fraction of the histones.

Because the EDTA and high salt extracts have fewer nonhistone proteins than the whole nuclei have, the two-dimensional electrophoretograms of the extracts are more easily interpreted. These electrophoretograms still have a background which can be presumed to be caused by disulfide bonds involving H3 and a variety of nonhistone proteins. Individually, these oligomers are not sufficiently abundant to appear as distinct spots on the gel; together they create a blurriness in the picture. Nevertheless, in the various two-dimensional electrophoretograms, distinct spots in the streak of H3 can be seen to be vertically aligned with proteins A and E and with the group B, C, and D. These results confirm the association of these five nonhistone proteins with histone H3.

The difference in the extractability of A as compared with B, C, D, and E is intriguing and may reflect some aspect of their localization within the nucleus. Electron microscopy shows the nucleus to be heterogeneous in appearance; the nucleolus is a distinct structure within the nucleus, and regions of diffuse and condensed chromatin can be distinguished as well. Undoubtedly, at an even finer level, there is further heterogeneity which may require a biochemist's tools for elucidation. As has been shown in immunofluorescence studies using polytene chromosomes from Drosophila salivary glands and antibodies against specific nonhistones, some proteins are found throughout the chromosomes while others are restricted in location (Silver & Elgin, 1978).

In earlier work from this laboratory (Grebanier & Pogo, 1979), protein A was seen to have certain distinctive characteristics. Its cross-linking after incubation with 2-iminothiolane was altered when the nuclei were treated with DNAase I first; cross-linking of other nonhistone proteins was not affected. Also, protein A was seen to be cross-linked to a much greater extent in nuclei from undifferentiated cells than in those from differentiated cells. In the present study, protein A is found to be the only disulfide cross-linked protein extracted with the nucleosomes. The selectivity of extraction may reflect some specialized structural or functional role of the protein.

At our present state of knowledge, however, it would be presumptive to attribute any particular functional significance to the properties of the proteins we have studied. Their proximity to histone H3 suggests that they may have important roles, and their differential extractability provides some hint of specialized localization. Clearly, much work remains to be accomplished before a full understanding will be reached.

A further question must be raised: which form or forms of histone H3 are involved in these disulfide bonds? In all of

the H3 variants found in the mouse, residue 110 is a cysteine, but in one variant a second cysteine is present at position 96 (Franklin & Zweidler, 1977). In a nucleosome, the two H3 molecules can be arranged so that the two cysteines at position 110 are close to each other (Camerini-Otero & Felsenfeld, 1977; Dietrich et al., 1978; Zama et al., 1978). Our observation of the ready formation of the H3–H3 homodimer by simple oxidation of nuclei could be taken as evidence to support this aspect of a model of the positioning of histones within the nucleosome. To account for the formation of disulfide bonds between H3 and the nonhistone proteins, one could postulate either that the cysteine at position 96 in one H3 variant is involved or else that bonds can form between nonhistones and the cysteine at position 110 on molecules of H3 which are in special conformations. Unfortunately, we have not yet been able to answer this question because of technical difficulties. The nonhistone proteins have not been solubilized under conditions appropriate for electrophoretic separation of the histone variants. Further, the impossibility of adding reducing reagents during preparation of our cross-linked samples can only confuse the issue by causing an increase in the number of apparent variants (Zweidler, 1978).

One observation would tend to support the involvement of the cysteine at position 96, but the evidence is by no means conclusive. On the two-dimensional electrophoretograms of the EDTA or salt extracts, we see relatively large amounts of protein running on the diagonal that correspond in mobility to the off-diagonal spots A–D. The on-diagonal protein has not been cross-linked, even when H_2O_2 has been added to ensure oxidizing conditions. Yet, the nonhistone protein appears to be tightly associated to the histone, since it is extracted together with the histone and appears together with it on sucrose gradients. Possibly, the nonhistone may be bound to the histone and the association stabilized by the formation of a disulfide bond when the histone H3 has a cysteine at position 96.

That many of these disulfide bonds may exist within the intact cell is interesting. Of the proteins we have discussed, only protein E is not bound by a disulfide bond if the nuclei are isolated in the presence of iodoacetate; the other cross-links are found under these conditions. Thus, the disulfide cross-linking we have been studying may occur naturally.

Added in Proof

In recent experiments we reduced the potassium iodoacetate concentration to 2.5 mM.

Acknowledgments

We thank Dr. Loren Day for his helpful comments on our work and Lillian Keyes and Paula Krauss for their assistance in preparing this manuscript.

References

Bakayev, V. V., Bakayeva, T. G., & Varshavsky, A. J. (1977) *Cell 11*, 619.

Bakayev, V. V., Bakayeva, T. G., Schmatchenko, V. V., & Georgiev, G. P. (1978) *Eur. J. Biochem. 91*, 291.

Berezney, R., & Coffey, D. S. (1974) *Biochem. Biophys. Res. Commun. 60*, 1410.

Camerini-Otero, R. D., & Felsenfeld, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5519.

Dieterich, A. E., Axel, R., & Cantor, C. R. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 199.

Franklin, S. G., & Zweidler, A. (1977) *Nature (London) 266*, 273.

Friend, C., Scher, W., Holland, J. G., & Sato, T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 378.

Goodwin, G. H., Sanders, C., & Johns, E. W. (1973) *Eur. J. Biochem. 38*, 14.

Grebanier, A. E., & Pogo, A. O. (1979) *Cell 18*, 1091.

Jackson, J. B., Pollock, J. M., & Rill, R. L. (1979) *Biochemistry 18*, 3739.

Kornberg, R. D. (1974) *Science (Washington, D.C.) 184*, 868.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Levy-W., B., Connor, W., & Dixon, G. H. (1979) *J. Biol. Chem. 254*, 609.

Long, B. H., Huang, C.-Y., & Pogo, A. O. (1979) *Cell 18*, 1079.

Mathew, C. G. P., Goodwin, G. H., & Johns, E. W. (1979) *Nucleic Acids Res. 6*, 167.

Miller, T. E., Huang, C.-Y., & Pogo, A. O. (1978) *J. Cell Biol. 76*, 675.

Silver, L. M., & Elgin, S. C. R. (1978) *Chromosoma 68*, 101.

Thomas, C. A., Jr., Saigo, K., McLeod, E., & Ito, J. (1979) *Anal. Biochem. 93*, 158.

van Holde, K. E., Sahasrabuddhe, C. G., & Shaw, B. R. (1974) *Nucleic Acids Res. 1*, 1579.

Vidali, G., Boffa, L. C., & Allfrey, V. G. (1977) *Cell 12*, 409.

Wang, K., & Richards, F. M. (1974) *Isr. J. Chem. 12*, 375.

Zama, M., Bryan, P. N., Harrington, R. E., Olins, A. L., & Olins, D. E. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 31.

Zweidler, A. (1978) *Methods Cell Biol. 17*, 223.