Trischitta, V., Gullo, D., Squatrito, S., Pezzino, V., Goldfine, I. D., & Vigneri, R. (1986) *J. Clin. Endocrinol. Metab. 62*, 522–528.
Trowbridge, M., & Draznin, B. (1986) *Horm. Metab. Res. 18*, 156–158.
Ueda, M., Robinson, F. W., Smith, M. M., & Kono, T. (1985) *J. Biol. Chem. 260*, 3941–3946.
Wang, C.-S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.
White, M. F., Takayama, S., & Kahn, C. R. (1985) *J. Biol. Chem. 260*, 9470–9478.
White, M. F., Stegmann, E. W., Dull, T. J., Ullrich, A., & Kahn, C. R. (1987) *J. Biol. Chem. 262*, 9769–9777.
Yoshimasa, Y., Seino, S., Whittaker, J., Kakehi, T., Kosaki, A., Kuzuya, H., Imura, H., Bell, G. I., & Steiner, D. F. (1988) *Science 240*, 784–787.
Zick, Y., Kasuga, M., Kahn, C. R., & Roth, J. (1983) *J. Biol. Chem. 258*, 75–80.

The DNase I Sensitive State of "Active" Globin Gene Chromatin Resists Trypsin Treatments Which Disrupt Chromatin Higher Order Structure†

Martha Lundell‡ and Harold G. Martinson*
Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received May 2, 1989; Revised Manuscript Received July 28, 1989

ABSTRACT: Active genes in higher eukaryotes reside in chromosomal domains which are more sensitive to digestion by DNase I than the surrounding inactive chromatin. Although it is widely assumed that some modification of higher order structure is important to the preferential DNase I sensitivity of active chromatin, this has so far not been tested. Here we show that the structural distinction between DNase I sensitive and resistant chromatin is remarkably stable to digestion by trypsin. Chick embryonic red blood cell nuclei were subjected to increasing levels of trypsin digestion and then assayed in the following three ways: (1) by gel electrophoresis for histone cleavage, (2) by sedimentation and nuclease digestion for loss of higher order structure, and (3) by dot-blot hybridization to globin and ovalbumin probes for disappearance of preferential DNase I sensitivity. We have found that chromatin higher order structure is lost concomitantly with the cleavage of histones H1, H5, and H3. In contrast, the preferential sensitivity of the globin domain to DNase I persists until much higher concentrations of trypsin, and indeed is not completely abolished even by the highest levels of trypsin we have used. We therefore conclude that the structural distinction of active chromatin, recognized by DNase I, does not reside at the level of higher order structure.

Active genes in higher eukaryotes reside in chromosomal domains which are more sensitive to digestion by DNase I than the surrounding inactive chromatin [Weintraub & Groudine, 1976; reviewed in Reeves (1984)]. This DNase I sensitive state generally encompasses both the active genes themselves and the nontranscribed regions of chromatin flanking them (Alevy et al., 1984; Jantzen et al., 1986; Lawson et al., 1982; Scott et al., 1987). This is in contrast to various transcriptionally induced perturbations of chromatin which have been characterized and which are limited both temporally and spatially to the regions actually undergoing transcription (Allegra et al., 1987; Chen & Allfrey, 1987; Cohen & Sheffery, 1985; Dorbic & Wittig, 1987; Johnson et al., 1987; Moreno et al., 1986; Smith et al., 1984; Wu & Simpson, 1985). The transcriptionally induced perturbations probably represent additional structural alterations beyond those which are responsible for the DNase I sensitivity of the large DNase I sensitive domains which almost always extend beyond the transcription units themselves.

Speculation regarding the structure of active chromatin has focused on three classes of chromatin component: the H1 histones (which includes H5), the core histone amino-terminal segments, and the two smaller high mobility group (HMG)[1] proteins. Both the H1 histones and the core histone amino-terminal segments have been implicated in the maintenance of higher order structure in chromatin [Allan et al., 1986; Böhm & Crane-Robinson, 1984; Hilliard et al., 1986; Klingholz & Strätling, 1982; Morse & Cantor, 1986; reviewed in Reeves (1984)], and elimination or modification of these components is generally presumed to relax higher order structure and facilitate chromatin activity (Nelson et al., 1986; Reeves, 1984; Ridsdale & Davie, 1987; Rocha et al., 1984; Weintraub, 1984). The two small HMG proteins have an unknown structural role but appear to be specific components of active chromatin (Dorbic & Wittig, 1987; Brotherton & Ginder, 1986; Reeves, 1984; Rocha et al., 1984).

Although it is clear that the H1 histones are important determinants of higher order structure, the vagaries of higher order structure itself leave the exact role played by H1 unclear (Felsenfeld & McGhee, 1986; Pederson et al., 1986). Three properties of chromatin are attributable to the H1 histones: the dinucleosomal periodicity of chromatin (Klingholz & Strätling, 1982; Thoma & Koller, 1981), the folding of the elementary nucleosomal filament into the 30-nm fiber [reviewed in Reeves (1984)], and aggregation (Jin & Cole, 1986). Active regions of chromatin are frequently proposed to be partially depleted in H1 (Reeves, 1984; Ridsdale & Davie, 1987; Rocha et al., 1984), to be associated with particular H1

† This work was supported by U.S. Public Health Service Grant GM35750.
* Author to whom correspondence should be addressed at the Department of Chemistry and Biochemistry, University of California.
‡ Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

[1] Abbreviation: HMG, high mobility group.

0006-2960/89/0428-9757$01.50/0

subtypes (Roche et al., 1985; Delabar, 1985), or to be associated with H1 in a special way (Weintraub, 1984; Ridsdale et al., 1988). Regardless of the details, it seems almost certain that active chromatin is not condensed into the same kind of stable, H1-dependent superstructure as inactive chromatin.

Although active chromatin and inactive chromatin presumably differ in higher order structure, it is not clear how, or even whether, these differences contribute to the difference detected by DNase I between the two chromatin types. The fact that fully condensed mitotic chromosomes retain preferential sensitivity in the regions which were active during interphase (Gazit et al., 1982) suggests that differences in higher order structure are not of major importance. Direct evidence contrary to a role for H1 in maintaining DNase I sensitivity has been obtained from studies in which H1 removal from nuclei failed to affect the level of preferential sensitivity of active regions to DNase I digestion (Goodwin et al., 1985; Villeponteau et al., 1978). Reports that preferential DNase I sensitivity of active sequences is retained even in excised nucleosomes lacking H1 would also appear to question any role for H1 or higher order structure in maintaining DNase I sensitivity (Giri & Gorovsky, 1980; Reeves, 1984). However these results have not proven to be reproducible and are controversial (Goodwin et al., 1985; Reeves, 1984). Goodwin et al. (1985), who found preferential DNase I sensitivity in H1-depleted nuclei but not in isolated nucleosomes, have suggested that the presence of H1 per se is not necessary but that some aspect of higher order structure, possibly unrelated to the presence of H1, is nevertheless required.

Some of these apparent discrepancies may be resolved by the report of Villeponteau et al. (1984), who showed that in chick erythrocyte nuclei micrococcal nuclease cleavage of chromatin leads to loss of DNase I sensitivity of active sequences over a wide range of nuclease treatment. Thus, the degree of preferential sensitivity depends on the level of micrococcal nuclease digestion used to prepare the nucleosomes and may reflect multiple features which differ in their ability to survive loss of higher order structure.

The structural role of the core histone amino-terminal tails in chromatin is even less clear than the role of H1. However, as for H1, most data point to a role in chromatin higher order structure (Böhm & Crane-Robinson, 1984; Hilliard et al., 1986; Morse & Cantor, 1986; Reeves, 1984). Also, similar to the situation for H1, modifications of these tails are usually prominent attributes of active chromatin (Allegra et al., 1987; Brotherton & Ginder, 1986; Johnson et al., 1987; Nelson et al., 1986; Reeves, 1984; Ridsdale & Davie, 1987; Rocha et al., 1984). The most prevalent modification is acetylation and virtually all histone acetylation occurs in these tail domains (Böhm & Crane-Robinson, 1984). Chromatin which has been hyperacetylated, by treatment of cells with butyrate, is severalfold more sensitive than normal chromatin to digestion by DNase I [see Reeves (1984)]. These correlations suggest a direct role for acetylation in the establishment of the DNase I sensitive state of chromatin. However, Brotherton and Ginder (1986) have reported that acetylation, unlike DNase I sensitivity, is not an obligatory feature of active chromatin.

HMG proteins 14 and 17 have been reported to confer DNase I sensitivity directly on active chromatin in vitro. However, the significance of this observation remains uncertain (Reeves, 1984), and the results are difficult to reproduce (Goodwin et al., 1985; Reeves, 1984). Nevertheless, recent data continue to indicate a role of some kind for the HMGs as legitimate constituents of active chromatin (Dorbic & Wittig, 1987; Brotherton & Ginder, 1986; Rocha et al., 1984).

At the present time, it seems clear that altered interactions with H1, multiple acetylations on the histone amino-terminal tails, and the presence of HMG proteins 14 and 17 are all genuine attributes of active chromatin. However, it remains unclear how, or whether, these features may contribute to those aspects of altered structure which are directly responsible for maintaining the preferential sensitivity to DNase I of active chromatin in isolated nuclei.

Previously, we examined the role of the DNA in the maintenance of the DNase I sensitive property of active chromatin in vivo (Villeponteau & Martinson, 1987). Here we address the role of the histones, and of higher order structure, in preserving this state in isolated nuclei in vitro. We have used trypsin to remove segments of H1 (and H5) and the core histones from chick embryo red blood cell nuclei. This inactivates the two major determinants of chromatin higher order structure without physically separating the nucleosomes and without use of harsh treatments such as acid extractions or high-salt washes. We show that H1, H5, and the core histone tails can be cleaved, and higher-order structure lost, at levels of trypsin digestion which are 1–2 orders of magnitude less than that which causes loss of preferential DNase I sensitivity in the active β-globin gene domain.

Materials and Methods

Trypsin Digestion of Nuclei. Twelve-day chick embryo red blood cell nuclei were isolated as previously described (Villeponteau et al., 1984), washed once in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 30% glycerol, and 0.3 M sucrose, and then resuspended in the same buffer at a concentration of 650 μg of DNA/mL. Trypsin (Sigma), dissolved in the same buffer, was added to give the concentrations indicated in the figures, and the mixture was incubated at 37 °C for 15 min. Digestion was stopped by addition of a 2-fold weight excess of soybean trypsin inhibitor (Worthington) followed by incubation for 30 min at 37 °C.

Protein Analysis. Samples were chilled, brought to 0.4 N H_2SO_4 or 5% $HClO_4$, left on ice for 25 min, and then centrifuged at 12000*g* for 5 min. Histones were precipitated from the supernatants using acetone and were analyzed by electrophoresis on 18% sodium dodecyl sulfate, polyacrylamide gels with staining by Coomassie blue. The bands were quantitated by densitometry.

Nuclease Digestions and Hybridization Analysis. DNase I (Sigma) digestions were for 4 min at 37 °C. For micrococcal nuclease (Sigma), the samples were made 0.5 mM in $CaCl_2$, and digestion was for 10 min at 37 °C. To compensate for the increased accessibility of the DNA in trypsin-digested chromatin, the amounts of nuclease used were varied according to the extent of trypsin predigestion of the samples (see figure legends). Nuclease digestions were terminated by making the samples 0.2% in sodium dodecyl sulfate and 15 mM in EDTA. Standard techniques for DNA isolation and blot hybridization analysis were used (Feinberg & Vogelstein, 1983; Villeponteau et al., 1984). Autoradiograms of the blots were quantitated by densitometry. DNA size was determined by electrophoresis on 1.2% agarose gels with staining by ethidium bromide.

Results

Preferential DNase I Sensitivity of Active Chromatin Survives Trypsin Digestion. In our hands, a typical DNase I sensitivity assay of chick embryo red blood cell nuclei gives the results shown in the first two columns of the dot blot shown in Figure 2A. Digestion of the nuclei with DNase I (column 2), but not with micrococcal nuclease (column 1), selectively eliminates globin, compared to ovalbumin, sequences in the

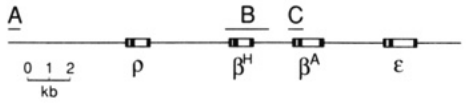

FIGURE 1: Hybridization probes from the β-globin domain used in this study. The map is taken from Villeponteau et al. (1982) with some revisions. Probe A is an *Eco*RI fragment. Probe B is a *Bam*HI fragment. Probe C is a *Hin*fI fragment.

DNA. A quantitative representation of the results is shown in the histogram below the dot blot.

Predigestion of the nuclei with up to 0.05 μg/mL trypsin (followed by trypsin inhibitor) prior to the DNase I step has no effect on the preferential DNase I sensitivity of the globin gene (Figure 2A, columns 3–5) despite considerable degradation of the histones (Figure 2, panels B and C, lane 5). Figure 2B shows an electrophoretic analysis of the total acid-extracted histones from each sample. Over half of the H1 and H5 in the 0.05 μg/mL trypsin sample (lane 5) has been cleaved, leaving a trail of degradation products. Considerable core histone cleavage has also occurred in this sample, evidenced by the presence of the degradation products, P1–P5, throughout a broad region below the H2A band. (The band below H4 in lane 2, at zero trypsin, is residual globin protein.) At this level of degradation, it is unlikely that any nucleosome has escaped attack by trypsin.

The degree of H1 and H5 degradation was also examined by using $HClO_4$ extraction which is specific for H1, H5, and the HMGs (Figure 2C). Unfortunately, cleavage of the HMGs is difficult to assess at this level of analysis because the HMGs are surrounded by H1 and H5 degradation products in the gel. For H1 and H5, however, it is again clear that 0.05 μg/mL trypsin (lane 5) leads to extensive degradation with no effect on preferential DNase I sensitivity.

Further predigestion of the nuclei with 0.1–0.3 μg/mL trypsin leads to an apparent loss of DNase I sensitivity (Figure 2A, columns 6–8) associated with further degradation of the histones (Figure 2B,C). However, at this level of histone degradation, the chromatin begins to undergo extensive, visible aggregation which interferes with DNase I digestion. The apparent loss of preferential sensitivity in this experiment (Figure 2A, column 8) therefore probably reflects the inaccessibility of the chromatin to DNase I. Nevertheless, even at 0.15 μg/mL trypsin (lane 7), a concentration at which most of the histones have been cleaved, preferential sensitivity remains greater than 3-fold, and even this slight loss in apparent preferential sensitivity may be accounted for by inaccessibility to DNase I of less than one-fourth of the sample due to aggregation. Agarose gel analysis of the DNA confirms that a large fraction of the DNA is cleaved poorly by the DNase I in samples with visible chromatin aggregation (data not shown).

Average curves summarizing the data from three experiments, including that of Figure 2, are shown in Figure 3. It is clear that protein cleavage commences at concentrations of trypsin which are several-fold lower than those which initiate loss of DNase I sensitivity. However, the precipitous loss of DNase I sensitivity between 0.1 and 0.3 μg/mL trypsin (solid curve) is almost certainly an artifact of the extensive aggregation which occurs in those samples and probably does not represent a meaningful trypsin-induced change in DNase I sensitivity. From the early portions of the curves, we therefore conclude that considerable protein cleavage (i.e., up to at least 50%) can occur with little or no accompanying effect on preferential DNase I sensitivity.

In an attempt to minimize the problem of aggregation, we have repeated the above experiment in the presence of polyglutamate. Polyglutamate has been shown to reduce H5-mediated aggregation of reconstituted nucleohistone (Stein & Bina, 1984), and preliminary experiments demonstrated that it reduced visible aggregation in our trypsin-digested chromatin as well (data not shown). Unfortunately, concentrations of polyglutamate which were sufficient to eliminate completely aggregation of the DNase I digested chromatin also reduced the ability of the DNase I to discriminate between active and inactive sequences. We therefore chose a slightly suboptimal concentration of polyglutamate which alleviated aggregation sufficiently to allow handling of the samples but did not

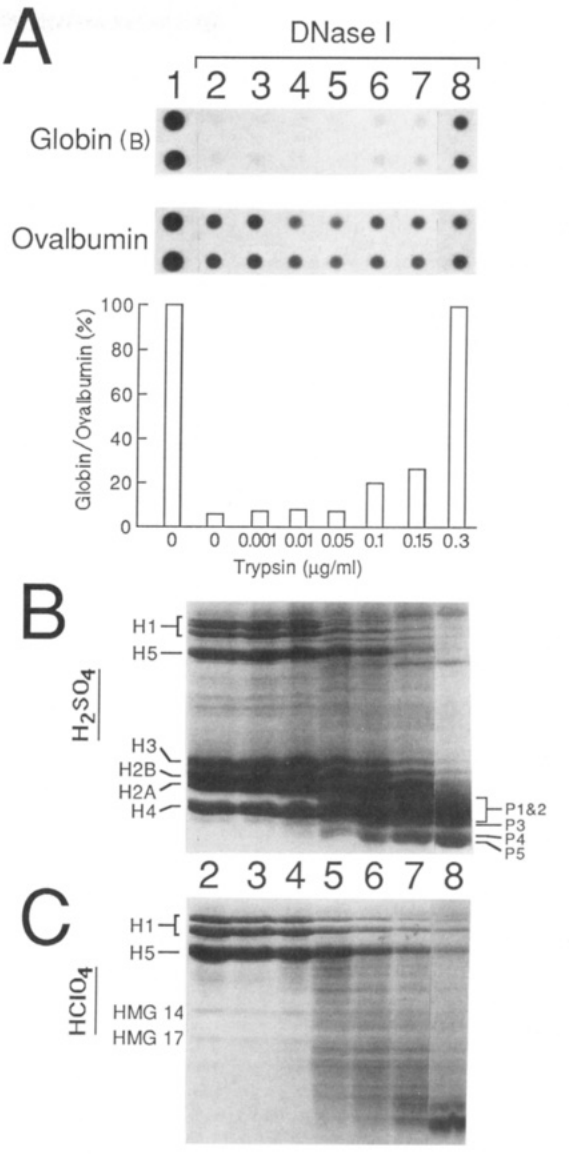

FIGURE 2: Effect of trypsin digestion of nuclei on the histone proteins and on globin gene DNase I sensitivity. (A) For columns 2–8, nuclei were treated with the concentrations of trypsin indicated below the histogram and then digested with 34 μg/mL DNase I. DNA was isolated from a portion of each sample, and 2 μg was spotted in duplicate on two identical dot blots for hybridization either to globin probe B or to the ovalbumin cDNA probe, pOV230, from McReynolds et al. (1977). Column 1 is a control sample of DNA from micrococcal nuclease digested nuclei which we used in normalizing the data as previously described (Villeponteau et al., 1984). To prepare the histogram, each hybridization signal for the DNase I treated samples was first expressed as a fraction of the signal in column 1 and then as the ratio of globin/ovalbumin. (B and C) Additional portions of each DNase I digested sample were extracted with H_2SO_4 or $HClO_4$ and analyzed on polyacrylamide gels. The diffuse band above the H1 region is DNase I. P1–P5 are the degradation products of the core histones (Böhm & Crane-Robinson, 1984). The gels have been cropped to show only the region in which the histones migrate.

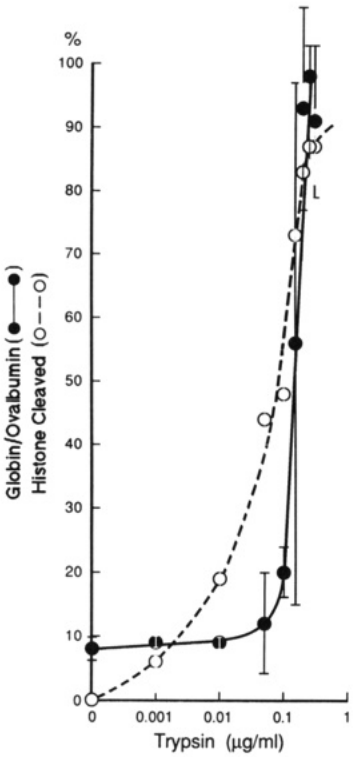

FIGURE 3: Summary of trypsin effects on the histone proteins and on globin gene DNase I sensitivity. A quantitative summary of the data in Figure 2, averaged with two additional experiments, is presented. To calculate the extent of trypsin cleavage, the protein bands to be quantitated were first expressed as a fraction of the total intensity of the protein in the lane, and then the normalized intensities of the bands in the trypsin lanes were subtracted from the similarly normalized intensities of the corresponding bands in the lanes for zero trypsin. The values thus obtained for H3, H5, and both bands of H1 were averaged for each experiment and then over the three experiments. The error bars show $\pm\sigma$, the standard deviation, over the three experiments (or over two experiments where data points do not exist for all three). All quantitations were of H_2SO_4-extracted proteins. No attempt has been made to quantitate the cleavage of H2A, H2B, or H4 because of the difficulties in quantitation and the contribution of degradation products to that region of the gel. More points are plotted on the graph of Figure 3 than are shown in the illustrative gels of Figure 2. The DNase I values were calculated as described for Figure 2 and then averaged as for the protein.

eliminate it altogether. We then carried out numerous DNase I digestion experiments at this polyglutamate concentration. All experiments led to the same general conclusion, but we selected for quantitative analysis only those experiments in which the DNase I exhibited greater than a 9–10-fold preference for globin over ovalbumin sequences at zero trypsin concentration.

Figure 4 shows the results from such a trypsin–DNase I experiment carried out in the presence of polyglutamate to reduce aggregation. In this experiment, the course of DNase I digestion was monitored by using two widely separated hybridization probes from the β-globin domain (probes A and C; see Figure 1). The data from this experiment and two others are summarized in Figure 5. Although there is increased scatter in the hybridization data at the higher trypsin concentrations owing to residual trypsin-induced aggregation (discussed below), the hybridization curves for both probes A and C are nevertheless essentially the same. Moreover, the main interpretation is from the early part of the curves where the data are most reliable and which show, in both cases, that histone cleavage (Figure 5, dashed line) precedes the loss of preferential DNase I sensitivity (solid or dotted lines) by well over an order of magnitude in trypsin concentration. This

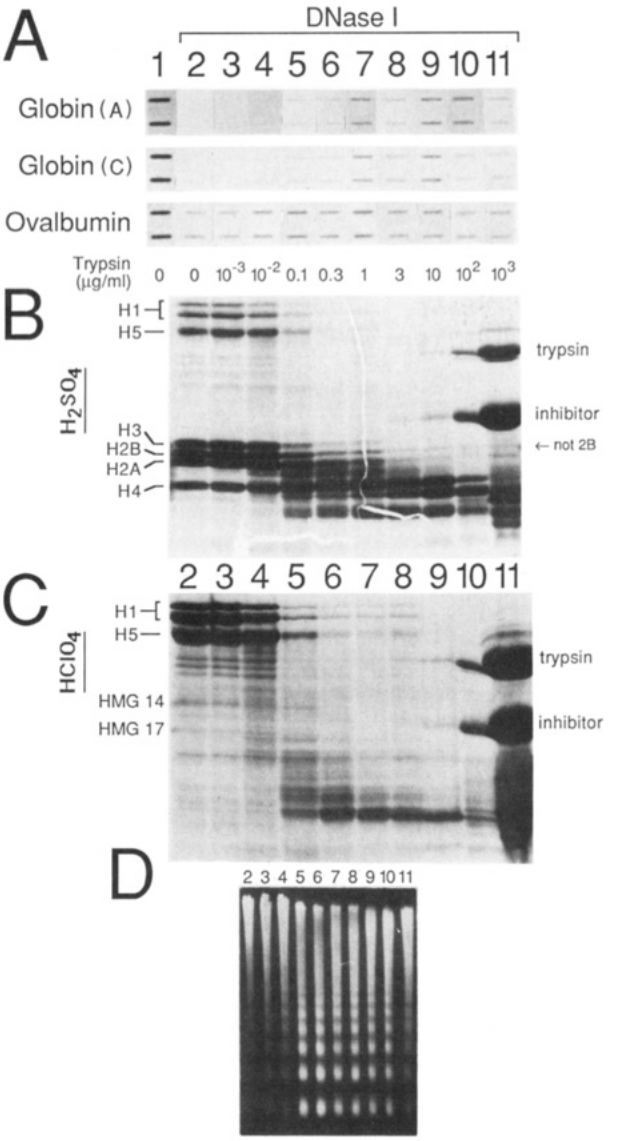

FIGURE 4: Extensive trypsin digestion of nuclei in the presence of polyglutamate. Panels A–C show the results of an experiment similar to that of Figure 2 except that 325 μg/mL polyglutamate (Sigma) was added to the nuclei, followed by a 10-min incubation at 37 °C, prior to the trypsin digestion step. In this experiment, the samples for protein analysis were aliquoted prior to the addition of DNase I. The following DNase I concentrations were used: lanes 2–4, 7 μg/mL; lanes 5 and 6, 3.5 μg/mL; lanes 7 and 8, 1.8 μg/mL; and lanes 9–11, 0.5 μg/mL. Lane 1 is purified undigested chicken DNA. The arrow on the right points to the position of an exogenous marker protein added in error to these samples. Panel D shows a micrococcal nuclease digest of an aliquot of each trypsin-digested sample. The following nuclease concentrations were used: lanes 2–4, 30 units/L; lanes 5 and 6, 15 units/L; lanes 7 and 8, 7.5 units/L; lanes 9–11, 3.7 units/L. The autoradiographic exposures shown in panel A were chosen so as to yield a relatively consistent ovalbumin signal in columns 2–11. The exposure within any given column is constant.

agrees with our conclusions from Figure 3 for which yet another hybridization probe was used (probe B, Figure 1). These equivalent results for widely separated hybridization probes confirm that we are monitoring the *domain* DNase I sensitivity of the globin region.

In the experiments of Figures 4 and 5, trypsin concentrations of up to 1 mg/mL were used. At the highest of these trypsin concentrations, degradation of the histones to their trypsin-resistant core sequences (Böhm et al., 1981; Allan et al., 1980) is virtually complete, and the HMG proteins are gone (Figure 4B,C, lanes 10 and 11). Yet micrococcal nuclease digestion reveals that the native nucleosomal organization of the chro-

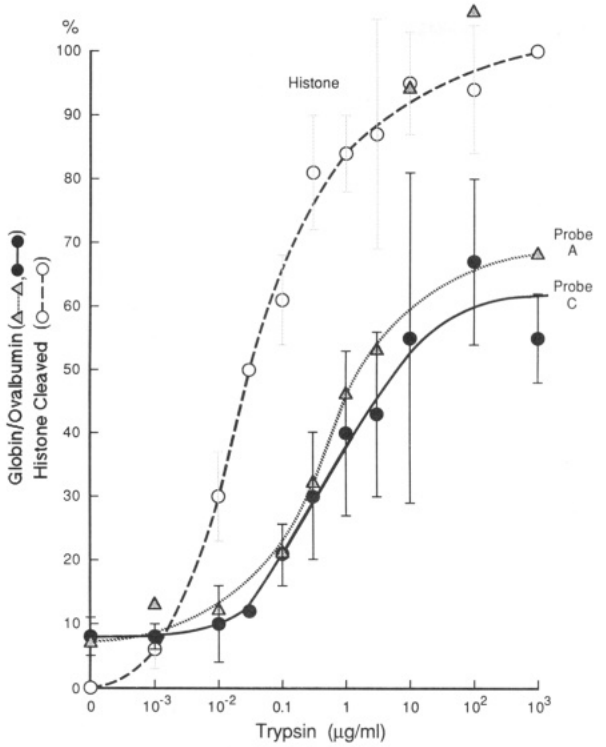

FIGURE 5: Summary of results from extensive trypsin digestion. The data from Figure 4 and two additional experiments are summarized in the same way as for Figure 3. The curve for probe C (●) is an average for all three of the experiments. The curve for probe A is just from the experiment of Figure 4, the only experiment for which both probes A and C were used.

matin remains intact (Figure 4D), in agreement with the previous report of Allan et al. (1982). This trypsinized "core chromatin" is completely soluble and therefore free from any aggregation-dependent DNase I digestion artifacts. DNase I digestion of this highly trypsinized core chromatin has consistently exhibited retention of a residual level of preferential DNase I sensitivity for sequences in the globin domain (Figure 5, 10^3 μg/mL points).

This residual sensitivity, which survives 1 mg/mL trypsin, apparently reflects a fundamental difference between active and inactive chromatin within the very core of the nucleosome. However, we considered the possibility that, since much protein had been stripped away from the DNA, the preferential sensitivity which remained could reflect an intrinsic preference of DNase I for certain DNA sequences within the globin domain compared to sequences in the ovalbumin gene, or, as another alternative, the sensitivity remaining after extensive trypsin treatment could reflect a greater tendency for trypsin to digest chromosomal proteins away from active regions than from inactive regions, thus exposing the active regions preferentially to nucleases.

We have evaluated the first of these alternatives by digesting naked DNA with DNase I. We found that DNase I in fact has a slight preference for ovalbumin sequences rather than globin sequences, which rules out the first alternative (data not shown). We evaluated the second alternative by digesting trypsinized and untrypsinized nuclei with micrococcal nuclease. If trypsin were clearing proteins preferentially from active sequences, then we would expect to be able to detect the increased accessibility of these regions using micrococcal nuclease. Instead, we have found the reverse. An initial mild preference of micrococcal nuclease for active regions is diminished or lost upon trypsin treatment (data not shown). We therefore conclude that the increased sensitivity of active chromatin to DNase I reflects differences which include structural alterations at the very heart of the nucleosome which survive massive doses of trypsin.

Figure 5 (solid and dotted lines) thus reveals two components of preferential DNase I sensitivity in active chromatin. All DNase I sensitivity survives considerably more trypsin digestion than that sufficient for cleaving the histones. Not until trypsin concentrations are used which are an order of magnitude greater than that required for histone cleavage is there loss of the major component of DNase I sensitivity. However, the remaining 1.5–2-fold level of preferential sensitivity survives the highest levels of trypsin concentration we have tested.

As indicated earlier, the concentration of polyglutamate which we have used is not quite sufficient to prevent aggregation completely. The conclusions of the previous paragraph are therefore minimal conclusions since, as discussed earlier, aggregation presumably prevents adequate access of the DNase I to the chromatin. The effect of this residual aggregation is reflected in the large error bars for probe C in Figure 5 between 1 and 100 μg/mL trypsin. It is also evident in the out-lying points for probe A. The return to partial preferential sensitivity at the aggregation-free trypsin concentration of 10^3 μg/mL shows that the apparent loss of preferential sensitivity for probe A at 10 and 100 μg/mL is spurious (otherwise, the interpretation would have to be that 10^3/μg/mL trypsin leads to *reestablishment* of preferential DNase I sensitivity). It therefore appears likely that the loss of the major component of preferential DNase I sensitivity which occurs between 0.1 and 100 μg/mL trypsin in Figure 5 (probes A and C), though delayed in comparison to Figure 3, may still be artifactually premature despite the use of polyglutamate. This emphasizes the surprisingly limited effect of histone cleavage on preferential DNase I sensitivity.

Loss of Chromatin Higher Order Structure Precedes Loss of DNase I Sensitivity. The fact that histone cleavage precedes loss of preferential DNase I sensitivity during trypsin digestion suggests that maintenance of a distinction between active and inactive chromatin at the level of higher order structure is not an important factor in the recognition of active chromatin by DNase I. After making this observation in our early experiments, we decided to include direct assays for the presence of higher order structure in subsequent work.

Figure 6 shows the results of a simple sedimentation test for higher order structure carried out in conjunction with the experiment of Figure 4 (probe A of Figure 5). Following trypsin predigestion and addition of trypsin inhibitor, but before addition of DNase I, an aliquot of each nuclear suspension was centrifuged briefly at low speed to determine the trypsin concentration at which nuclear disruption, caused by loss of higher order structure, would render the nuclei nonsedimentable. (Trypsin-induced chromatin aggregation, when it occurs, gives rise to a gellike network which does not pellet during the brief low-speed spins used here.) The pellets and supernatants were then acid extracted for histone and analyzed by gel electrophoresis. Lanes 2–4 of Figure 6 show that chromatin continues to be recovered in the pellet after treatment with up to 10^{-2} μg/mL trypsin. In contrast, for trypsin concentrations of 0.1 μg/mL and above, the chromatin is recovered predominantly in the supernatant (Figure 6, lanes 5–11). Thus, a dramatic change in nuclear chromatin structure takes place in the range of 0.01–0.1 μg/mL trypsin. Comparison to Figure 5 shows that this transition corresponds directly to histone cleavage (dashed curve) but precedes the DNase I transition.

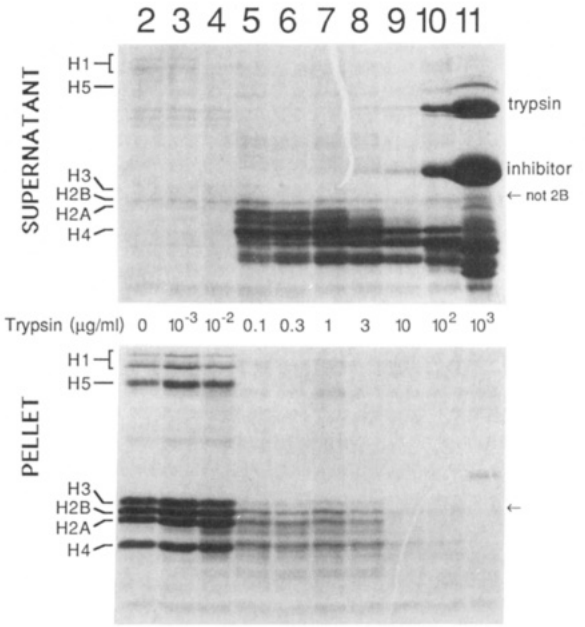

FIGURE 6: Low-speed sedimentation analysis of trypsin-digested nuclei. Aliquots were taken from samples 2–11 of Figure 4, following the trypsin digestion step, and centrifuged at 2000*g* for 6 min. The pellets were resuspended, and histones were extracted from both pellets and supernatants with 0.4 N H_2SO_4. Volumes representing equivalent amounts of starting material were loaded on the gels for both the pellet and the supernatant fractions. The arrows on the right point to the position of the exogenous marker protein.

In order to obtain a more quantitative measure of the trypsin-induced loss of higher order structure, we have taken advantage of the ability of DNase I to detect a dinucleosomal periodicity in condensed, but not in extended, chromatin (Klingholz & Strätling, 1982). The dinucleosomal periodicity of intact nuclei digested mildly with DNase I is illustrated in lane 1 of Figure 7A and in scan 1 of Figure 7B. It is characterized by the predominance of nucleosomal peaks at even-numbered positions. Predigestion of the nuclei with increasing concentrations of trypsin prior to the DNase I step, according to our normal experimental format, leads to progressive replacement of the dinucleosomal periodicity with a micrococcal nuclease like mononucleosomal periodicity (Figure 7, samples 2–8). Thus, we can quantitate the loss of higher order structure by quantitating the increase in size of the odd-numbered compared to the even-numbered nucleosomal peaks from DNase I digested, trypsin-treated nuclei.

The data of Figure 7 on the trypsin-induced loss of higher order structure, together with data on histone cleavage and on the globin to ovalbumin hybridization ratio following DNase I digestion of the same trypsin-treated nuclei, are summarized in Figure 8A. The loss of higher order structure is expressed in Figure 8A as the appearance of a mononucleosomal periodicity in the DNase I digestion pattern (see legend to Figure 8). The curves show that the loss of higher order structure in chromatin (i.e., the increase in mononucleosomal periodicity) is closely correlated with the cleavage of H1, H5, and H3 as expected, but is not correlated with the loss of preferential DNase I sensitivity which occurs much later.

The correlation of loss of higher order structure with histone cleavage but not with loss of DNase I sensitivity is absolutely consistent. It has been observed in several independent experiments and occurs whether polyglutamate is added before or after digestion with trypsin. Scans of DNA gels from three additional experiments are shown in Figure 8B. In each case, it is clear that the mononucleosomal repeat pattern begins to dominate at 0.1 μg/mL trypsin in agreement with the ex-

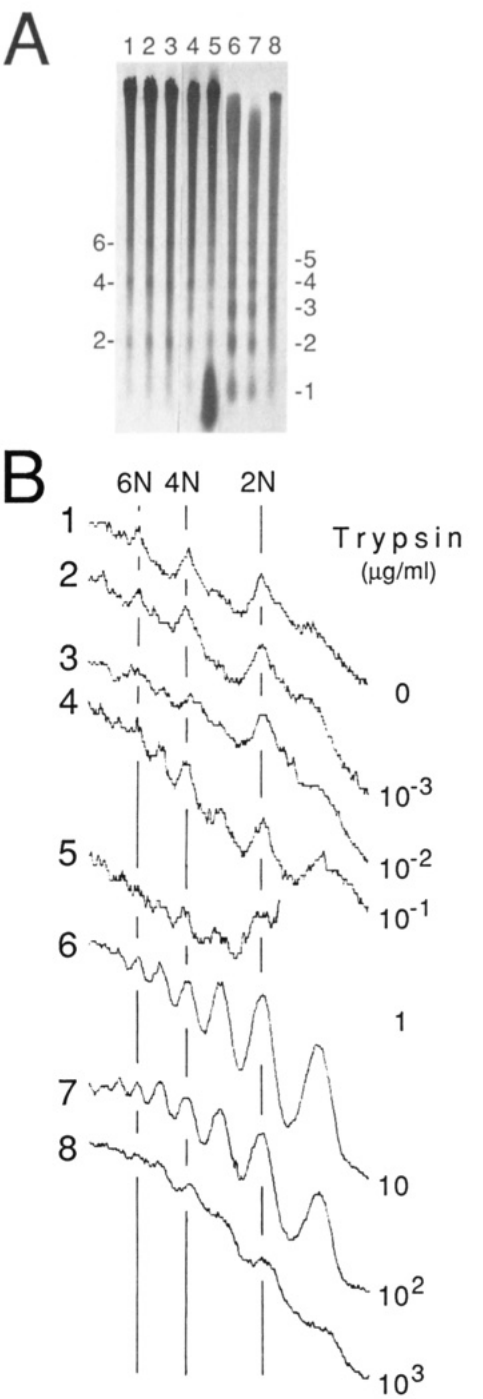

FIGURE 7: Trypsin-induced transition from dinucleosomal to mononucleosomal periodicity in chromatin. Nuclei were digested with trypsin, polyglutamate was added to give 325 μg/mL, and then DNase I digestion was carried out using the following concentrations of DNase I: lanes 1–5, 7 μg/mL; lanes 6–8, 0.7 μg/mL. The gel in panel A was scanned to give the tracings in panel B.

periment of Figure 7. In all cases, histone quantitation confirms that the curves for histone cleavage and for loss of the dimer periodicity coincide (curves not shown). Drinkwater et al. (1987) have reported that for some tissues the ratio of monosomal to disomal periodicity varies with the extent of DNase I digestion. We have examined this in some experiments by quantitating the dinucleosomal → mononucleosomal transition for several extents of DNase I digestion. The correlation of histone cleavage and loss of dinucleosomal periodicity is unchanged regardless of the extent of DNase I digestion.

From these results, we conclude that the determinants of

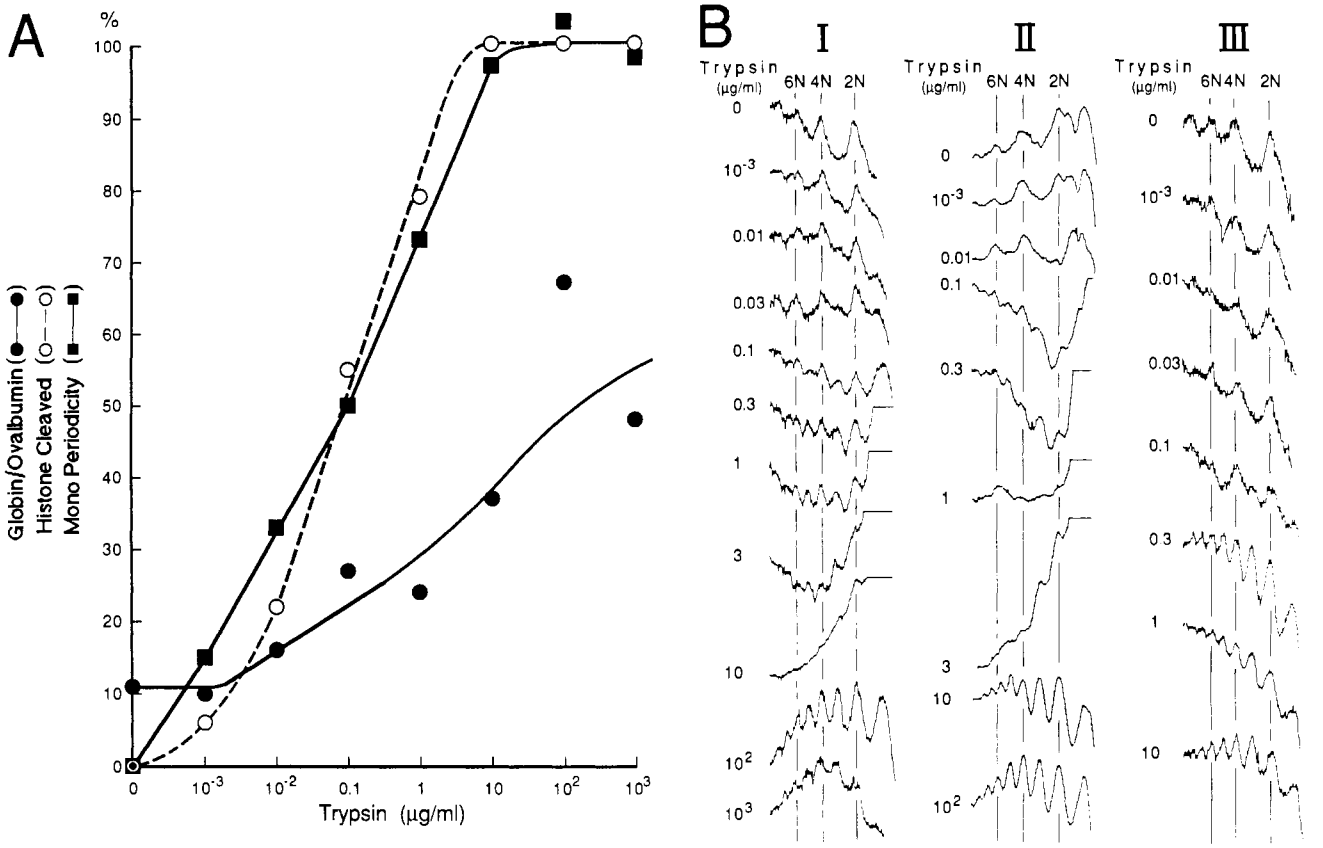

FIGURE 8: Loss of dinucleosomal periodicity is correlated with histone cleavage but not with loss of preferential DNase I sensitivity. The data of Figure 7 were part of a complete experiment which is summarized by the curves shown in part A. The transition from dinucleosomal to mononucleosomal periodicity is represented quantitatively by a measure of the appearance of the odd-numbered trinucleosome and pentanucleosome peaks. The appearance of these peaks was quantitated individually as the difference of their heights and the average of the heights of the flanking even-numbered peaks. This difference was then normalized to that of the zero-trypsin sample on a percentage basis and subtracted from 100. The trinucleosome and pentanucleosome values so obtained for each sample were then averaged. Finally, all values were further normalized to the average for samples 6 and 7 which were considered to be 100%. For sample 5, which was overdigested by DNase I, only the pentasome value was used. The globin sequence used for hybridization in this experiment was probe C. The hybridization control was purified undigested chicken DNA. In part B, panels I and II present the scans of the DNase I digested samples which, together with the samples of Figure 7, represent the three experiments summarized in Figure 5. Panel III is from an additional experiment carried out solely to confirm, using optimized DNase I digestion conditions (and a 5-min rather than a 30-min incubation in trypsin inhibitor), the relationship between histone cleavage and the loss of the dinucleosome repeat. As for all the other experiments, the midpoint of the histone cleavage curve from this experiment coincides with the midpoint of the monosomal periodicity curve calculated from the scans of panel III (curves not shown).

DNase I sensitivity do not depend on any obvious aspects of chromatin higher order structure but reside instead within the structure of the nucleosomes themselves.

Discussion

We have used trypsin to dissect the β-globin chromatin of red blood cell nuclei. On the basis of the criterion of resistance to trypsin digestion, we find three levels of organization in chromatin. The first level, the most sensitive to trypsin, is that of chromatin higher order structure. The loss of higher order structure accompanies the cleavage of the histones at low concentrations of trypsin. The second level of organization, which is less sensitive to trypsin, is that which is characteristic of the major component of preferential DNase I sensitivity of active β-globin chromatin. The loss of this component of DNase I sensitivity comes at higher concentrations of trypsin, following histone cleavage and the loss of chromatin higher order structure. The third level of organization, resistant to the highest levels of trypsin we have employed, is characterized by a low but definite residual degree of preferential sensitivity to DNase I in the β-globin domain.

Chromatin Higher Order Structure. By two entirely different criteria, chromatin higher order structure appears to be the first casualty of trypsin digestion. The first criterion, sedimentability of nuclei at low speed (Figure 6), probably reflects the H1-specific aggregation of chromatin studied by Jin and Cole (1986). The results of Figure 6, together with those of Figure 5, show a close correspondence between the cleavage of histones H1, H5, and H3 and the loss of chromatin sedimentability. H1 is known to cause chromatin aggregation in Mg^{2+}-containing buffers of the type we use here (Jin & Cole, 1986), and H3 probably also contributes in accord with its proposed role in assisting the H1-mediated condensation of chromatin (Ajiro & Nishimoto, 1985; Mazen et al., 1987). It is noteworthy that the histone cleavage curves of Figure 3, 5, and 8 reflect only the initial cuts introduced by the trypsin into the histone polypeptide chains. Thus, complete histone integrity is required for the histone-specific aggregation of chromatin in Figure 6 to occur. This supports the view of Jin and Cole (1986) that H1-mediated aggregation of chromatin (as opposed to aggregation of trypsinized chromatin) is a meaningful function of the H1 histones.

The second criterion for higher order structure that we have employed is the presence of the dinucleosomal periodicity in chromatin as recognized by DNase I (Burgoyne & Skinner, 1982; Klingholz & Strätling, 1982; Pospelov & Svetlikova, 1982). This is a rigorous criterion for higher order structure because a dinucleosomal periodicity is the simplest recognizable structural element in chromatin above the level of the unstructured 10-nm nucleosomal filament. It is a property either

of the basic higher order 30-nm fiber itself or of a structural state intermediate between the 10-nm filament and the 30-nm fiber (Felsenfeld & McGhee, 1986; Sen et al., 1986; Thoma & Koller, 1981). Figure 8 demonstrates that the dinucleosomal periodicity of chromatin disappears in concert with the intact histones during the earliest stages of trypsin digestion.

In contrast to the strict correlation between histone cleavage and higher order structure (Figures 5–8), there is no such correlation between either of these parameters and preferential DNase I sensitivity (Figures 3, 5, and 8). If the principal attribute of DNase I sensitive chromatin were a deficiency in the ability of the histones to participate in higher order structure formation, then the elimination of higher order structure from the DNase I resistant chromatin via trypsin digestion should eliminate most of the distinction between the sensitive and resistant compartments. Since this is clearly not the case, we conclude that histone-mediated differences in higher order structure are not among the major structural distinctions between active and inactive chromatin recognized by DNase I in isolated nuclei. Of course, this does not imply that these distinctions do not exist, only that they are not the ones recognized by DNase I.

Major Component of Preferential DNase I Sensitivity. Following histone cleavage and loss of chromatin higher order structure, further digestion with much higher concentrations of trypsin removes most, but not all, of the distinction between active and inactive chromatin as recognized by DNase I. As with loss of chromatin higher order structure, loss of preferential sensitivity is a consequence of trypsin attack on bulk (i.e., inactive) chromatin. Trypsin treatment dramatically increases the overall DNase I digestion rate of nuclei (e.g., see legend to Figure 7), suggesting that in heavily trypsinized nuclei it is an increase in the rate of bulk (inactive) chromatin digestion rather than a decrease in the rate of active chromatin digestion which ultimately causes the two rates to become more similar.

Unfortunately, trypsin-induced aggregation in our samples has prevented us from determining the particular cleavage event responsible for the ultimate loss of preferential DNase I sensitivity. Aside from the unmanageable state of the sample itself, this aggregation is most obvious as an abrupt loss of preferential DNase I sensitivity (see Figure 3) which appears concomitantly with the visible aggregation. Aggregation may simply cause chromatin accessibility to be the rate-limiting factor in DNase I digestion, thus overriding any conformation-based differences in susceptibility to the enzyme. Alternatively, aggregation may precipitate widespread changes in chromatin conformation which obliterate the differences between active and inactive chromatin.

By use of polyglutamate, we were able to reduce aggregation sufficiently to establish that the DNase I sensitive conformation of active chromatin is not dependent on complete histone integrity or on folding of the elementary nucleosomal filament into a specific higher order structure (Figures 5 and 8). However, low levels of visible aggregation persisted at some of the higher (but not at the highest) trypsin concentrations. Thus, it is not clear whether the loss of DNase I sensitivity in the range of 1 μg/mL trypsin (Figures 5 and 8) is genuine or whether, as in Figure 3, it is artifactually premature. If it is premature, then the major structural distinction recognized by DNase I in active and inactive chromatin is far more resistant to trypsin digestion than suggested even by the data of Figure 5.

Residual Component of Preferential DNase I Sensitivity. After aggregation has been completely eliminated by digestion with very high concentrations of trypsin, a low level of residual preferential DNase I sensitivity can be seen still to persist (Figure 5, 10^3 μg/mL trypsin). The lack of any detectable change in the micrococcal nuclease digestion patern of this heavily digested chromatin (Figure 4D) shows that the dissection of chromatin with trypsin is an orderly process (despite the aggregation) and yields a genuine trypsin-resistant residue of the native structure rather than a random assembly of trypsin-resistant materials. We have carried out control experiments which show that the residual preferential DNase I sensitivity of the active chromatin is not the result either of an inherent preference of DNase I for globin sequences or of a tendency for trypsin to denude active sequences preferentially. Although Weintraub and Groudine (1976) have reported that trypsin does digest the histones of active chromatin preferentially, their experiments involved extensive micrococcal nuclease digestion of the nuclei prior to the trypsin step. Excision of nucleosomes from chromatin is known to affect the mode of trypsin attack (Böhm & Crane-Robinson, 1984). Their results therefore cannot be compared with the experiments reported here which are based on trypsin digestion of intact nuclei. We conclude that at least one component of the structural distinction between active and inactive chromatin recognized by DNase I resides within the trypsin-resistant core of the nucleosome.

The persistence of a structural distinction between the trypsinized cores of active and inactive chromatin could reflect the existence of a large activation energy barrier to interconversion between the two conformations, at least for cores. Alternatively, there may remain, even in the trypsin-resistant cores, compositional differences between DNase I sensitive and resistant nucleosomes. Since the HMG proteins and all of the histone segments bearing posttranslational modifications have been stripped away by the trypsin (Böhm & Crane-Robinson, 1984), virtually the only candidates remaining to account for compositional differences among the trypsinized nucleosome cores would be core histone variants. Recently, variant forms of H2A have attracted particular attention because of their enrichment in active chromatin regions (van Daal et al., 1988; Donahue et al., 1986; Ridsdale & Davie, 1987). Indeed, the most prominent distinguishing sequence features of the H2A variants, which are of probable functional importance (White et al., 1988), fall well within the trypsin-resistant domain of H2A (Böhm & Crane-Robinson, 1984). These variant sequences therefore definitely persist in trypsinized core chromatin and may contribute to the difference in conformation detected by DNase I.

The structural feature recognized by DNase I in trypsinized core chromatin is unlikely to be directly related to the structural change described by Allfrey and colleagues as being characteristic of actively transcribed chromatin (Allegra et al., 1987; Chen & Allfrey, 1987; Johnson et al., 1987). The structure described by these authors exists as a direct result of, and in direct proportion to, the degree of actual transcription (Chen & Allfrey, 1987). In contrast, Figure 5 of this report shows that two widely separated regions of the globin domain (probes A and C) are quantitatively very similar in their response to trypsin and DNase I digestion despite transcriptional levels that differ by at least 30-fold (Villeponteau et al., 1982).

Registry No. DNase I, 9003-98-9.

References

Ajiro, K., & Nishimoto, T. (1985) *J. Biol. Chem. 260*, 15379–15381.

Alevy, M. C., Tsai, M.-J., & O'Malley, B. W. (1984) *Biochemistry 23*, 2309–2314.
Allan, J., Hartman, P. G., Crane-Robinson, C., & Aviles, F. X. (1980) *Nature 288*, 675–679.
Allan, J., Harborne, N., Rau, D. C. & Gould, H. (1982) *J. Cell Biol. 93*, 285–297.
Allan, J., Mitchell, T., Harborne, N., Böhm, L., & Crane-Robinson, C. (1986) *J. Mol. Biol. 187*, 591–601.
Allegra, P., Sterner, R., Clayton, D. F., & Allfrey, V. G. (1987) *J. Mol. Biol. 196*, 379–388.
Böhm, L., & Crane-Robinson, C. (1984) *Biosci. Rep. 4*, 365–386.
Böhm, L., Briand, G., Sautière, P., & Crane-Robinson, C. (1981) *Eur. J. Biochem. 119*, 67–74.
Brotherton, T. W., & Ginder, G. D. (1986) *Biochemistry 25*, 3447–3454.
Burgoyne, L. A., & Skinner, J. D. (1982) *Nucleic Acids Res. 10*, 665–673.
Chen, T. A., & Allfrey, V. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5252–5256.
Cohen, R. B., & Sheffery, M. (1985) *J. Mol. Biol. 182*, 109–129.
Delabar, J. M. (1985) *J. Biol. Chem. 260*, 12622–12628.
Donahue, P. R., Palmer, D. K., Condie, J. M., Sabatini, L. M., & Blumenfeld, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4744–4748.
Dorbic, T., & Wittig, B. (1987) *EMBO J. 6*, 2393–2399.
Drinkwater, R. D., Wilson, P. J., Skinner, J. D., & Burgoyne, L. A. (1987) *Nucleic Acids Res. 15*, 8087–8103.
Feinberg, A., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6–13.
Felsenfeld, G., & McGhee, J. D. (1986) *Cell 44*, 375–377.
Gazit, B., Cedar, H., Lerer, I., & Voss, R. (1982) *Science 217*, 648–650.
Giri, C. P., & Gorovsky, M. A. (1980) *Nucleic Acids Res. 8*, 197–214.
Goodwin, G. H., Nicolas, R. H., Cockerill, P. N., Zavou, S., & Wright, C. A. (1985) *Nucleic Acids Res. 13*, 3561–3579.
Hilliard, P. R., Smith, R. M., & Rill, R. L. (1986) *J. Biol. Chem. 261*, 5992–5998.
Jantzen, K., Fritton, H. P., & Igo-Kemenes, T. (1986) *Nucleic Acids Res. 14*, 6085–6099.
Jin, Y., & Cole, R. D. (1986) *J. Biol. Chem. 261*, 15805–15812.
Johnson, E. M., Sterner, R., & Allfrey, V. G. (1987) *J. Biol. Chem. 262*, 6943–6946.
Klingholz, R., & Strätling, W. H. (1982) *J. Biol. Chem. 257*, 13101–13107.
Lawson, G. M., Knoll, B. J., March, C. J., Woo, S. L. C., Tsai, M.-J., & O'Malley, B. W. (1982) *J. Biol. Chem. 257*, 1501–1507.
Mazen, A., Hacques, M.-F., & Marion, C. (1987) *J. Mol. Biol. 194*, 741–745.
McReynolds, L. A., Catterall, J. F., & O'Malley, B. W. (1977) *Gene 2*, 217–231.
Moreno, M. L., Chrysogelos, S. A., Stein, G. S., & Stein, J. L. (1986) *Biochemistry 25*, 5364–5370.
Morse, R. H., & Cantor, C. R. (1986) *Nucleic Acids Res. 14*, 3293–3310.
Nelson, D. A., Ferris, R. C., Zhang, D., & Ferenz, C. R. (1986) *Nucleic Acids Res. 14*, 1667–1682.
Pederson, D. S., Thoma, F., & Simpson, R. T. (1986) *Annu. Rev. Cell Biol. 2*, 117–147.
Pospelov, V. A., & Svetlikova, S. B. (1982) *FEBS Lett. 146*, 157–160.
Reeves, R. (1984) *Biochim. Biophys. Acta 782*, 343–393.
Ridsdale, J. A., & Davie, J. R. (1987) *Nucleic Acids Res. 15*, 1081–1097.
Ridsdale, J. A., Rattner, J. B., & Davie, J. R. (1988) *Nucleic Acids Res. 16*, 5915–5926.
Rocha, E., Davie, J. R., van Holde, K. E., & Weintraub, H. (1984) *J. Biol. Chem. 259*, 8558–8563.
Roche, J., Gorka, Cl., Goeltz, P., & Lawrence, J. J. (1985) *Nature 314*, 197–198.
Scott, M. J., Tsai, M.-J., & O'Malley, B. W. (1987) *Biochemistry 26*, 6831–6840.
Sen, D., Mitra, S., & Crothers, D. M. (1986) *Biochemistry 25*, 3441–3447.
Smith, R. D., Yu, J., & Seale, R. L. (1984) *Biochemistry 23*, 785–790.
Stein, A., & Bina, M. (1984) *J. Mol. Biol. 178*, 341–363.
Thoma, F., & Koller, Th. (1981) *J. Mol. Biol. 149*, 709–733.
van Daal, A., White, E. M., Gorovsky, M. A., & Elgin, S. C. R. (1988) *Nucleic Acids Res. 16*, 7487–7497.
Villeponteau, B., & Martinson, H. G. (1987) *Mol. Cell Biol. 7*, 1917–1924.
Villeponteau, B., Lasky, L., & Harary, I. (1978) *Biochemistry 17*, 5532–5536.
Villeponteau, B., Landes, G. M., Pankratz, M. J., & Martinson, H. G. (1982) *J. Biol. Chem. 257*, 11015–11023.
Villeponteau, B., Lundell, M., & Martinson, H. (1984) *Cell 39*, 469–478.
Villeponteau, B., Pribyl, T. M., Grant, M. H., & Martinson, H. G. (1986) *J. Biol. Chem. 261*, 10359–10365.
Weintraub, H. (1984) *Cell 38*, 17–27.
Weintraub, H., & Groudine, M. (1976) *Science 193*, 848–856.
White, E. M., Shapiro, D. L., Allis, C. D., & Gorovsky, M. A. (1988) *Nucleic Acids Res. 16*, 179–198.
Wu, T.-C., & Simpson, R. T. (1985) *Nucleic Acids Res. 13*, 6185–6203.