occurring well below the bilayer to hexagonal phase transition temperature indicates that Laurdan can also detect changes in membrane bilayer properties which are correlated with the tendency to form the hexagonal phase. The fact that the chemical and physical properties of Laurdan are different from DNS-Lys, yet both probes detect these changes in bilayer properties, gives further support that these probes are monitoring intrinsic properties of the membrane.

The results of the present paper demonstrate that bilayers with a high propensity for forming the hexagonal phase have markedly altered physical properties. Changes in the stability, hydrophobicity, and solvation of the bilayer surface may be responsible for the changes detected by the fluorescent probes. These alterations in membrane surface properties may also cause membrane functional changes which do not specifically require the formation of nonbilayer phase intermediates.

Registry No. DNS-Lys, 28217-24-5; DiPoPC, 56815-99-7; DiPoPE, 61599-23-3; Laurdan, 74515-25-6.

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Nucleosome Spacing Is Compressed in Active Chromatin Domains of Chick Erythroid Cells†

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ABSTRACT: We have cleaved the chromatin of embryonic and adult chicken erythroid cells using a novel nuclease that is capable of resolving clearly the nucleosomes of active chromatin. We found that in active chromatin, nucleosomes are spaced up to 40 base pairs closer together than in inactive chromatin. This was true for both "housekeeping" and "luxury" genes and was observed whether the digestion was carried out on isolated nuclei in vitro or by activating the endogenous nuclease in vivo. The close spacing extended several kilobases into flanking chromatin, indicating that this is a domain property of active chromatin, not just a characteristic of regions disrupted by transcription. A simple interpretation of our results is that the nucleosomes of active chromatin are mobile in vivo and, not being constrained by linker histones, freely move closer together.

Shortly after the discovery of the nucleosome as the fundamental subunit of chromatin (Kornberg, 1974), it was recognized that the average nucleosome periodicity varies widely from one organism and tissue to another (Eissenberg et al., 1985). The spacing between nucleosomes, averaged over the whole genome, is correlated with the overall degree of transcriptional activity of the cell. During the course of development, increasing transcriptional activity is correlated with decreasing internucleosomal repeat distance (Brown & Sutcliffe, 1987) whereas decreases in transcriptional activity are correlated with increases in the internucleosomal repeat distance (Chambers et al., 1983; Weintraub, 1978). For example, chicken erythroid cells, the subject of the present report, exhibit a steady increase in average nucleosome spacing throughout the genome during maturation (Weintraub, 1978). Focusing on vertebrate RNA polymerase II for our present discussion, several studies of specific genes also provide evidence consistent

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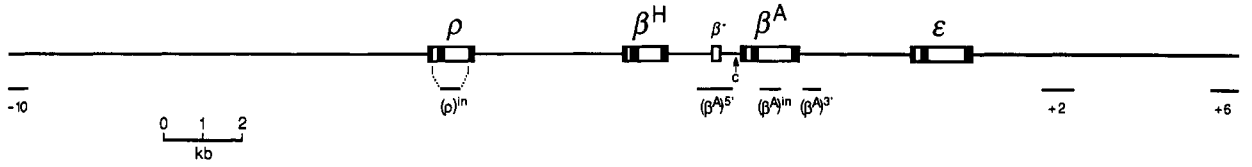

FIGURE 1: Globin domain hybridization probes used in this study. The map shows the four rightward-transcribing globin genes as well as the short, leftward-oriented, transcription unit, β^-, which lies between β^H and β^A (Pribyl & Martinson, 1988). The ρ^{intron} probe is a 510 bp fragment obtained by *Hae*III digestion of pCBG4 followed by *Bal*31 triming as shown in the figure [see Villeponteau and Martinson (1981) for a description of the clones]. The $\beta^{5'}$ and β^{intron} probes are 890 bp *Bam*HI–*Hin*fI and 517 bp *Hin*fI–*Hin*dIII fragments, respectively, from pCBG13. The $\beta^{3'}$ probe is a 460 bp *Hha*I fragment from pCBG15. The "−10" probe is a 500 bp *Eco*RI–*Hin*dIII fragment of unique-sequence DNA which lies more than 10 kb upstream of the globin gene cluster. The "+2" and "+6" probes are, respectively, pCBG21.6 and a *Hin*fI fragment from near the left end of pCBG26.

with this generalization (Balsquez et al., 1986; Moreno et al., 1986; Rose & Garrard, 1984; Stratling et al. 1986; Sun et al., 1986), but there are contrary reports as well (Gottesfeld & Melton, 1978; Smith et al., 1983), so no generalization about nucleosome spacing in active vs inactive chromatin is presently possible. However, it is clear that nucleosome spacing can vary between genes within a genome.

In our studies of chicken erythroid cells, we have asked whether nucleosome spacing depends merely on local influences such as the DNA sequence or the immediate effects of transcription, or whether it is a more broadly based, regional property of chromatin. Active genes exist within large chromatin domains, which include both transcribed and nontranscribed DNA, and which are characterized throughout by structurally altered nucleosomes that are more susceptible than bulk chromatin to digestion by DNase I (Alevy et al., 1984; Jantzen et al., 1986; Lawson et al., 1982; Weintraub & Groudine, 1976). These domains can encompass as much as 100 kilobase pairs of DNA and can include several related genes (Lawson et al., 1982; Villeponteau et al., 1984). If the spacing preferences of the altered DNase I sensitive nucleosomes are different from those of normal nucleosomes, or if any other parameter which impinges on spacing is altered in these domains, then we would expect that nucleosome spacing in the active chromatin domains would depart from the genomic average. During our work on the novobiocin-induced nuclease (Villeponteau et al., 1986), we noticed developmentally regulated changes in nucleosome spacing which appear to represent such a situation.

Here we describe the use of "novanase" (*novo*biocin-*a*ctivated *nuclease*) to study the problem of nucleosome spacing over several active and inactive genes and their flanking domains in various chicken cell types. We find, for chicken embryonic and adult erythroid cells, that the nucleosome spacing of chromatin surrounding active genes is shorter than that of bulk chromatin or of the chromatin of inactive genes. The spacing data suggest the existence of domain-wide influences on nucleosome spacing (or nucleosome mobility) which differ from the globally specified spacing (or mobility) parameters for the rest of the genome. In addition, closer examination of the data reveals that the nucleosomal spacing within active domains often consists of two discrete spacing modes, both shorter than that of the inactive chromatin.

Novanase has certain advantages over micrococcal nuclease for the study of nucleosome spacing. It has a blunt-ended, double-stranded mode of cleavage and does not have as strong a tendency to trim nucleosomes exonucleolytically or to cut within nucleosomes to yield subnucleosomal DNA (Villeponteau et al., 1986). These properties appear to contribute to the ability of novanase to recognize a nucleosomal repeat within actively transcribed genes where micrococcal nuclease yields only a smear. The cleavage reagent methidium-propyl-EDTA·Fe(II) also possesses the ability to recognize the positions of the structurally altered nucleosomes of actively transcribed genes (Benezra et al., 1986).

Materials and Methods

Preparation of Novanase Extract. Erythroid cells from adult chicken blood (Mission Labs, Rosemead, CA) or from 12-day chicken embryos (Villeponteau & Martinson, 1987) were pelleted and then resuspended in 20 volumes of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM NaH_2PO_4). The cells were prewarmed to 37 °C for 10 min, and then novobiocin (Sigma) was added to a final concentration of 1 mg/mL (Villeponteau et al. 1986). After 5 min at 37 °C, the cells were pelleted and then resuspended in phosphate-buffered saline without novobiocin for 5 min more. The cells were pelleted again and lysed by resuspending in 2 volumes of phosphate-buffered saline containing 0.5% NP 40. After homogenization by vortexing and vigorous pipeting, nuclei and debris were removed by a 3-min spin in a microcentrifuge. The supernatant was stored on ice for up to several hours or at −70 °C before use. Extracts were monitored to ensure freedom from endogenous DNA fragments.

Preparation and Digestion of Isolated Nuclei with Novanase. Erythroid cells were isolated from 5-, 12-, or 19-day embryos as above. The chicken lymphoid (MSB) cells (Akiyama & Kato, 1974) were grown on dishes in minimal essential medium containing 10% newborn calf serum. To prepare nuclei, cells were washed in phosphate-buffered saline, pelleted, and lysed for 1 min on ice in 40 volumes of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 40% glycerol, 0.3 M sucrose, and 0.4% NP 40. The nuclei were pelleted and resuspended in 4–6 volumes of novanase extract and incubated at 37 °C for 90 min. DNA was isolated as before (Villeponteau & Martinson, 1987).

In Situ Digestion of Nuclei with Endogenous Novanase in Intact Cells. Erythroid cells from 12-day embryos were treated with novobiocin as described above but were then subjected to a 90-min (rather than a 15-min) post-novobiocin incubation in order to allow the released novanase to act endogenously. Since novanase is not activated in all cells, cleaved and uncleaved chromatins were separated by the "supernovo" procedure (Villeponteau et al., 1986), and the DNA of the cleaved fraction was isolated as described above.

Hybridization. The DNA was electrophoresed on 1.2% agarose gels, Southern-blotted to nitrocellulose, and hybridized (Villeponteau & Martinson, 1981). The globin region probes used are shown in Figure 1. The ovalbumin cDNA clone is pOV230 (McReynolds et al., 1977). The conalbumin probe is the cDNA clone pBR322-con1 (Cochet et al., 1979). The glyceraldehyde-3-phosphate dehydrogenase probe (pG2 GAD 1.5) was kindly provided by Dr. Ming-Jer Tsai and covers 843 bp at the 5′ end of the gene plus 668 bp of 5′-flanking DNA. This clone is a derivative of one of the genomic clones described

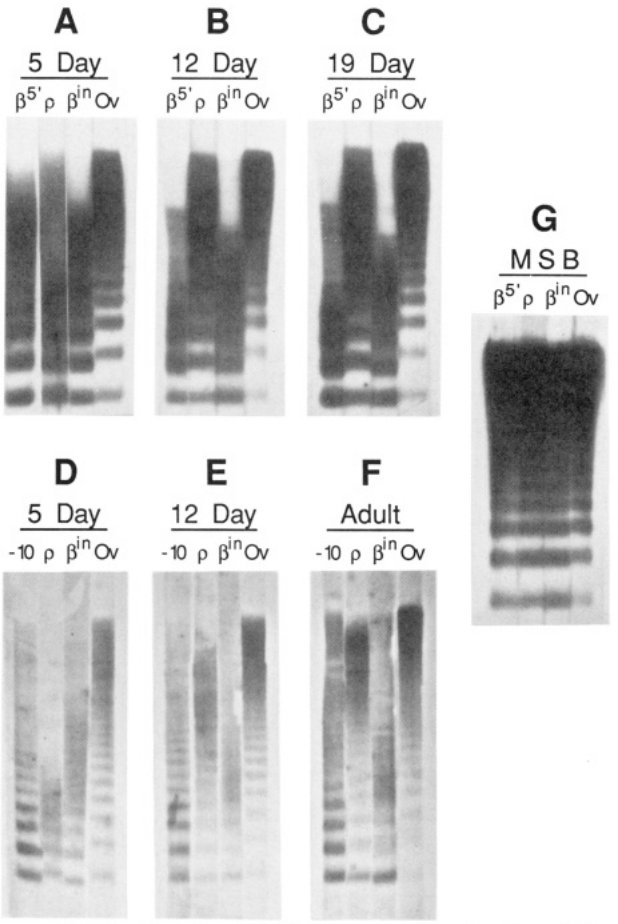

FIGURE 2: Digestion of nuclei with novanase extract in vitro. Extract from erythroid cells of 12-day embryos was used for panels A, B, C, and G. Extract from adult erythrocytes was used for panels D–F. As with novanase digestion in vivo (Villeponteau et al., 1986), digestion in vitro proceeds to a well-defined limit and then stops. All of the above are limit digests. For panels A, B, C, and G, DNA lengths were determined by reference to the ethidium bromide patterns which were calibrated using *Hin*dIII-cut phage λ DNA and *Hae*III-cut pBR322 DNA. For panels D–F, DNA lengths were determined by reference to *Hae*III-cut φX174 DNA which was included in each sample and hybridized and autoradiographed separately, using the same blots. DNA band positions were determined either by measurement of the autoradiograms directly or by mesurement of scans of the autoradiograms. For panels A, B, C, E, and F, the mobilities of the bands can be intercompared in the figure. The lanes from which panel D was assembled exhibited pronounced smiling and are not easy to intercompare in the figure; however, accurate molecular weight values for quantitative analysis were obtained from the internal markers.

in Alevy et al. (1984). For actin, we used the cDNA clone for β-actin (pA1) constructed by Cleveland et al. (1980). All probes are unique except for the β-actin probe which cross-hybridizes extensively among the various members of the actin gene family. The probes were labeled by nick-translation or random priming to a specific activity of (1–2) × 10^9 cpm/μg.

Results

Globin Gene Chromatin Possesses More Closely Spaced Nucleosomes than Ovalbumin Gene Chromatin in Chick Erythroid Cells. Nuclei from various chicken cell types were digested with the novobiocin-activated nuclease (novanase). The cleaved DNA was then electrophoresed, Southern-blotted, and hybridized to various probes. Panels A–C of Figures 2 and 3 show that in the erythroid cells of 5-, 12-, and 19-day embryos, globin gene chromatin has a shorter nucleosomal repeat length than ovalbumin gene chromatin. A similar but less pronounced effect is seen for erythrocytes from adult chickens (Figure 2F).

In contrast to erythroid cell chromatin, nonerythroid MSB cell chromatin possesses globin and ovalbumin gene regions having essentially the same repeat lengths (Figure 2, panel G). Thus, the nucleosomal periodicity of globin chromatin is tissue-specific.

To analyze the nucleosome spacing results quantitatively, we have plotted band number vs DNA length for each gel lane. Figure 4A shows the resulting plots obtained from the data of Figure 2A. The slope of each line gives the average nucleosome spacing over the region of the probe. The intercept gives the average amount by which the oligonucleosomes are either extended or trimmed at their termini. Systematic trimming by the nuclease will give plots with negative intercepts.

Figure 4A shows that, for 5-day erythroid cells, the average nucleosome spacing for all of the globin probes is less than that for ovalbumin. The lines converge toward zero, indicating little trimming by novanase. Plots of the 12-day and 19-day data give similar results.

Compressed Nucleosome Spacing Is a General Characteristic of Active Chromatin in Chick Erythroid Cells. The cell type-specific globin genes are not the only active genes to exhibit compressed nucleosome spacing in erythroid cells. Both of the housekeeping genes we have tested, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Figure 4B) and actin (Figure 5), do as well. Moreover, the actin cDNA probe we have used is actually homologous to the entire family of actins and thus represents several genes (Cleveland et al., 1980). Conversely, the longer spacing of the inactive ovalbumin gene chromatin appears to be representative generally of inactive sequences: the inactive conalbumin gene gives results which are indistinguishable from those for ovalbumin (Figures 5 and 6), and bulk chromatin (as detected by staining with ethidium bromide, not shown) exhibits long spacing as well.

Compressed Nucleosome Spacing Is a Domain Property of Active Chromatin. Figures 2 and 3 illustrate that the shortened repeat extends over the entire globin domain and is not limited to genes undergoing transcription. For example, in panel A of both figures, it can be seen that β has essentially the same short repeat length as ρ in 5-day erythroid cells (plotted in Figure 4A) although β is not transcribed in these cells (Groudine et al., 1981; Landes et al., 1982; Villeponteau et al., 1982). Conversely, Figure 2B,C shows that the ρ repeat is short in 12- and 19-day cells, which are of the definitive erythroid lineage and do not transcribe the ρ-globin gene [nor, most likely, do their precursors (Lois & Martinson, 1989)]. Thus the shorter repeat within the β-globin chromatin of the early embryo and within the ρ-globin chromatin of the late embryo reflects a property of the globin domain as a whole and is not merely a consequence of transcription per se.

The "−10" probe (Figure 1) also exhibits a shortened repeat (Figure 2D,E,F and Figure 4B) and shows that the domain of compressed spacing extends at least 10 kb upstream of the globin genes themselves. Using nuclear run-on transcription, we have not detected any transcription in the "−10" region (E. Erickson, unpublished data). We have obtained similar results (not shown) with the "+2" probe, an unrelated nontranscribed sequence in the 3′-flanking region (Figure 1). Thus, the domain of compressed nucleosome spacing encompasses more than 23 kb of chromatin.

The extent of chromatin cleavage, in contrast to the degree of spacing compression between nucleosomes, varies markedly

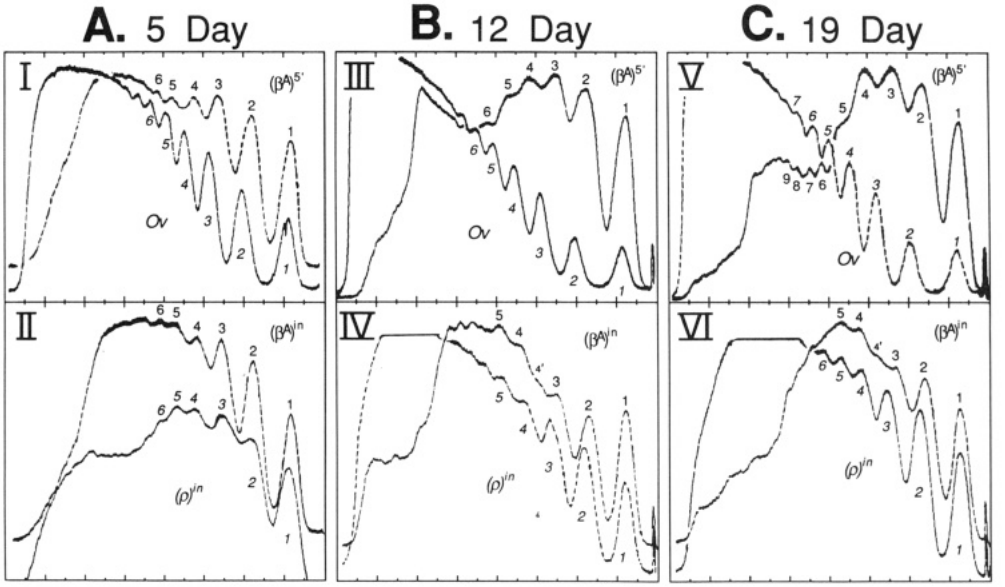

FIGURE 3: Densitometer scans of panels A–C of Figure 2.

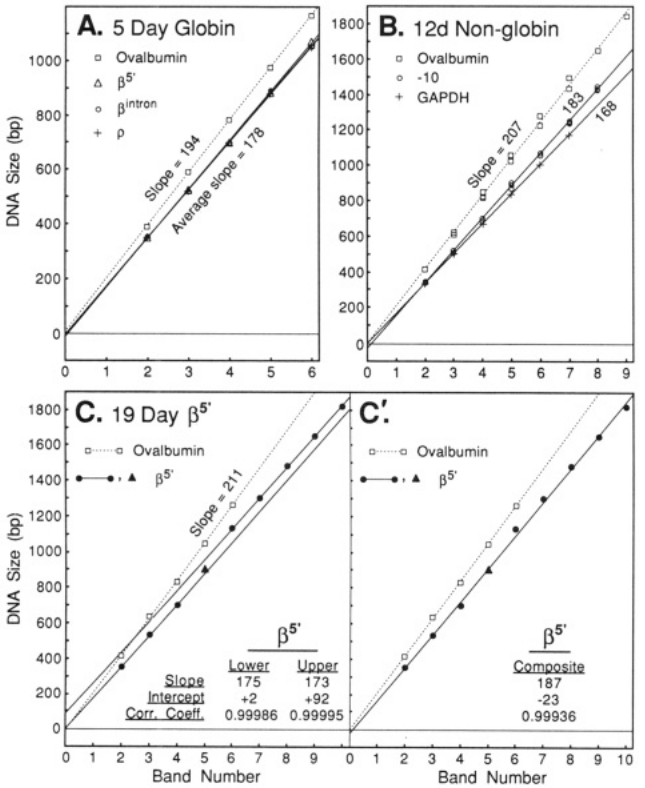

FIGURE 4: Linear regression analyses. Values for monomer are frequently aberrant and have been omitted from all of our analyses. Panel A shows plots of the data in Figure 3A. Panel B shows data from several experiments. The ovalbumin data are from Figure 2B,E. The "−10" data are from Figure 2E. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) data are from an experiment in which the GAPDH ladder was calibrated against an ovalbumin ladder in place of a separate set of standards. Panel C shows data from Figure 3C in which the lower and higher molecular weight $\beta^{5'}$ points are fitted to two different lines as discussed in the text. The shoulder at pentasome for $\beta^{5'}$ in Figure 3C was clearly in transition between the lower and higher molecular weight peaks and was not included in the regression calculations of panel C. Panel C′ shows the results of fitting a single line to all of the $\beta^{5'}$ points (including pentasome).

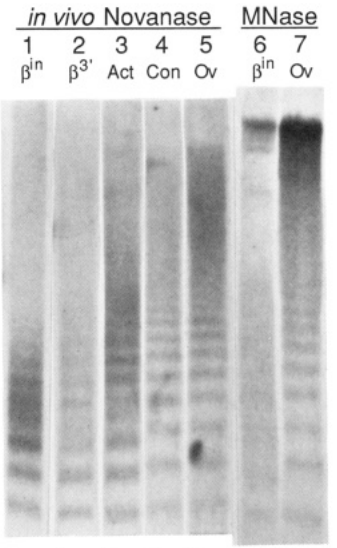

FIGURE 5: Digestion of erythroid cell nuclei from 12-day embryos in vivo with endogenous novanase or in vitro with micrococcal nuclease. Lanes 1–5 show the results of a limit digest with novanase. Lanes 6 and 7 show the results of a 20-min digest with 1.25 units/mL micrococcal nuclease (Sigma) at 37 °C (Villeponteau et al., 1986). Lanes 1–5 and 6–7 come from different gel runs.

throughout the globin domain and appears to be a gene-specific, rather than a domain-wide, property of chromatin. The extent of cleavage by novanase probably depends in part on the local rate of transcription, as reported previously for micrococcal nuclease digestion (Smith, R. D., et al., 1983, 1984). This is most easily seen in panels D–F of Figure 2 which show that the ρ gene is cleaved more rapidly than the β^A gene in nuclei from 5-day erythroid cells, but more slowly than the β^A gene in nuclei from 12-day or adult erythroid cells. This is correlated with the known regulation of transcription of these genes in which the ρ gene is transcribed at 5 days but not at 12 days or in the adult, whereas the β^A gene is transcribed at 12 days and in the adult (Affolter et al., 1987), but not at 5 days. However, other factors also govern the rate of nuclease cleavage as shown by the rapid rate of cleavage of the nontranscribed "−10" region (Figure 2D–F).

Statistical Positioning of Nucleosomes in the Globin Domain. The only nonzero intercept of significance that we have obtained from plots of our data is the *positive* intercept for bands 6–10 of the $\beta^{5'}$ probe in Figure 4C. These points fall on a line which is shifted relative to the line for bands 2–4 of the same probe. The intercept for the shifted line is +92, which means that all oligonucleosomes in the upper series are an average of about 90 bp longer than expected on the basis of nucleosome spacing alone (i.e., a deviation opposite to that

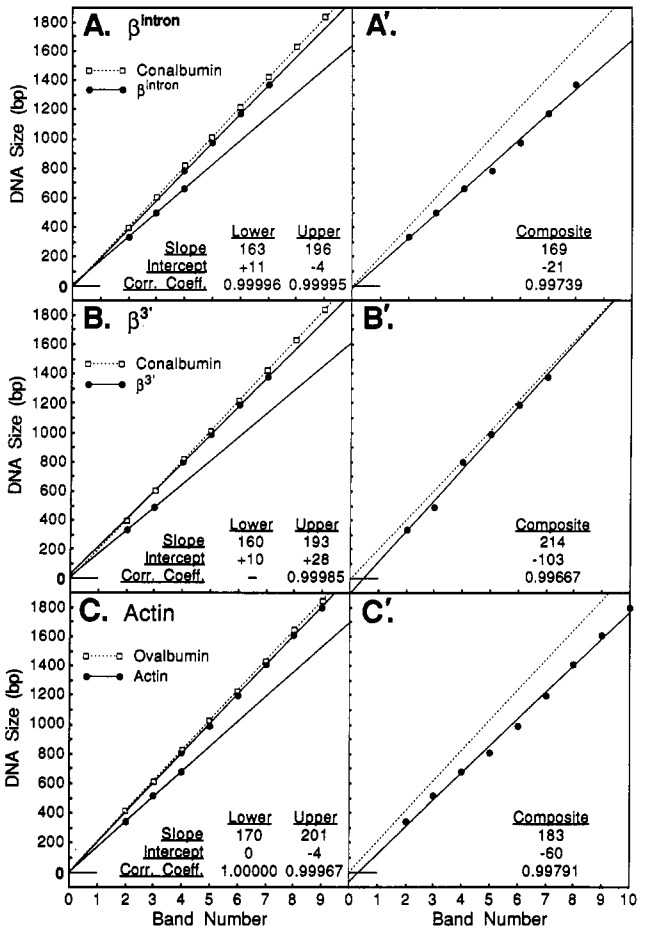

FIGURE 6: Linear regression analysis of the novanase data in Figure 5. In panels A–C, two lines of different slope are fitted to each set of active gene data points. In panels A′–C′, a single line is fitted to each set of points, and the two bands which were considered a doublet at position 4 in the case of panels A and C (see text) are plotted instead as separate bands in A′ and C′. For panels B and B′, the data points are plotted identically in the two panels because only one member of the doublet is clearly resolved in the gel (Figure 5, lane 2).

expected from nucleosome trimming). The analysis presented below suggests that this result is the consequence of statistical positioning of the nucleosomes (Kornberg & Stryer, 1988) next to a major hypersensitive site on the 5′ side of the β^A-globin gene. This site has been studied thoroughly by McGhee et al. (1981) and is cleaved efficiently by novanase (Villeponteau et al., 1986).

The data for Figure 4C come from panel C of Figure 2. In the $\beta^{5'}$ lane of Figure 2C, there is a sudden drop-off in intensity after the first four bands, but then the ladder pattern continues nonetheless to a high molecular weight. The bimodal nature of this pattern is obvious in the scan for this lane in panel V of Figure 3C. Panel III (Figure 3B) is similar, but the banding pattern of the upper series is not resolved.

In Figure 4C′, we have fitted a single line to all of the 19-day $\beta^{5'}$ points. It is clear that this single line does not represent the data as well as do the two lines of Figure 4C (e.g., compare the correlation coefficients). This result and the obviously bimodal nature of the gel pattern have persuaded us that the upper and lower sets of points in Figure 4C belong to two separate series.

We interpret the bimodal nature of the $\beta^{5'}$ pattern at 12 and 19 days to be the combined result of the particular position of the hybridization probe and the chromatin structure characteristic of the actively transcribed β^A gene in older embryos. After the first week of development, transcription of β^A (and β^-; Figure 1) is the most active of the entire region (Landes et al., 1982). Thus, compared to the ρ gene (Figure 2B,C,E) or the β^H gene (see below), cleavage in and around β^A and β^- is more complete, yielding primarily short oligonucleosomes. A plot of band number vs DNA length for these short oligonucleosomes extrapolates to near zero as described above. However, the $\beta^{5'}$ probe lies adjacent to a hypersensitive region, in front of the β^A gene ("C" in Figure 1), which is present in the chromatin of erythroid cells following the first week of development (McGhee et al., 1981). Virtually complete cutting by novanase takes place within this region [site C in Figure 6B of Villeponteau et al. (1986)]. Consequently, all long oligonucleosomes from the region between β^H and β^A, which happen to escape the frequent internucleosomal cutting in the vicinity of β^-, are foreshortened by a cut at C. This converts the experiment into a partial indirect end-label situation for the longer oligonucleosomes, which are thus bounded on the right by a cut at site C and on the left by a normal internucleosomal cut (see map in Figure 1). Therefore, our interpretation is that the first four bands in the lane correspond to a conventional oligonucleosomal ladder for the extensively cleaved region encompassed by the probe but that the faint bands higher in the lane correspond primarily to oligonucleosomes extending from site C into the β^H gene where cutting is much less frequent.

The circumstance that the $\beta^{5'}$ lane of Figure 2C is, to a large extent, an indirect end-label experiment allows a simple explanation for the positive intercept in Figure 4C: if the cut at site C is about 90 bp to the right of where the normal internucleosomal cut would be, then all oligonucleosomes bounded by site C will be 90 bp longer than expected. The very existence of a ladder in an indirect end-label situation such as this shows that the nucleosomes over the β^H gene are positioned relative to the hypersensitive site at C.

Two Discrete Alternative Spacing Modes Exist for Active Chromatin Domains. The scans for the $(\beta^A)^{intron}$ probe in panels IV and VI of Figure 3 illustrate another unusual feature in our data. Unlike the special case described above, this feature is common to many of the nucleosome ladders we have obtained for active chromatin. Comparison of the β^{intron} scans with the respective ρ^{intron} scans beneath them shows that the first three peaks of the β scans are more closely spaced than the peaks in the ρ scans, as is clearly evident from the bands in Figure 2. In contrast, peaks 4 and 5 of the β scans (panels IV and VI of Figure 3) appear to coincide with the ρ scans. Within the large separation between peaks 3 and 4 of the β^{intron} scans, there is a shoulder, labeled 4′.

The unusual appearance of the β^{intron} scans suggests the existence of a composite pattern consisting of long-periodicity chromatin fragments, in which higher multimers predominate, combined with short-periodicity chromatin fragments in which short oligomers predominate. This result suggests that both repeat patterns are represented in the chromatin and that the chromatin with the short repeat is cleaved to a greater extent than the chromatin with the long repeat.

Lanes 1–3 of Figure 5 show additional examples of the bimodal spacing phenomenon. In this experiment, chromatin was cleaved in situ within the intact cells, and the interrupted ladders are resolved to higher molecular weights. Plots of the data (Figure 6A–C) confirm, quantitatively, the existence of two series of chromatin fragments which differ in nucleosome spacing. The points fall clearly on two separate lines which share a common intercept but which differ in slope. The slope of the lower series is in the range characteristic of active chromatin (Figure 4). The slope of the upper series approaches

that for inactive chromatin but remains reproducibly less.

The improved resolution in this experiment allows the region of 4–4′ to display a clear doublet of bands in lanes 1 and 3 of Figure 5 rather than a band and a shoulder as in the situation discussed above for Figures 2 and 3. As shown in Figures 6A,C, one member of the doublet at 4 clearly belongs to the lower series and the other to the upper series.

Panels A′–C′ show the results of attempting to fit a single line to all of the points as if they comprised a single series. The points do not fit well on a single line. In fact, without exception, when an interruption is visible by eye on the autoradiogram, the resulting data points are fit well by two lines and poorly by a single line.

Compressed Nucleosome Spacing of Active Chromatin Is Not an Artifact of Nuclear Isolation, of the Use of Novanase, or of Excessive Digestion. We have examined nucleosome spacing not only by digestion of nuclei with novanase in vitro (Figures 2–4, and additional experiments) but also by activation of endogenous novanase in vivo (Figures 5 and 6). Thus, the data obtained both from intact cells and from isolated nuclei lead to the same conclusions: nucleosome spacing in active chromatin is compressed, and two distinct spacing modes are often observed.

Novanase was chosen for this study because it is capable of recognizing the nucleosomal nature even of transcriptionally active genes. It frequently has been observed that cleavage of chromatin with micrococcal nuclease (MNase) does not yield a clearly resolved nucleosomal pattern over active gene sequences (Cohen & Sheffery, 1985; Kondo et al., 1987; Moreno et al., 1986; Rose & Garrard, 1984; Stratling et al., 1986; Wu & Simpson, 1985). This is illustrated in lanes 6 and 7 of Figure 5. However, careful examination of lane 6 (especially in the original autoradiogram) shows that even with micrococcal nuclease there is at least faint evidence of both reduced spacing and alternate spacing modes for the β^A-globin gene. The two bands visible at the bottom of lane 6 are more closely spaced than the ovalbumin bands of lane 7 and match the close spacing of the globin and actin bands in lanes 1–3. These two bands clearly belong to the short series. It is also possible to discern faint bands belonging to the long series. This micrococcal nuclease pattern, faint though it is, shows that the phenomena we have described are properties of chromatin which do not depend on the presence of novanase.

In the experiments described so far, limit digests with novanase have been used. Curiously, novanase digestion ceases while most of the chromatin is still of oligonucleosomal length. This is convenient because overdigestion is, consequently, never a problem. The novanase limit is highly reproducible, and the resulting oligonucleosomes cover a size range ideal for analysis. Nevertheless, we wished to confirm that the reduced spacing between nucleosomes of active chromatin did not develop during the course of our digestion. Figure 7 shows, for the closely spaced nucleosomes of the "−10" probe, that compressed nucleosome spacing is observed very early in digestion, when most of the "−10" chromatin remains of high molecular weight. Indeed, linear regression analysis gives nucleosome spacings of 170 and 182 bp, respectively, at 20 and 90 min of digestion. These results, as well as a similar analysis using a globin gene probe (not shown), provide no indication that the reduced nucleosome spacing of active chromatin develops during the course of digestion.

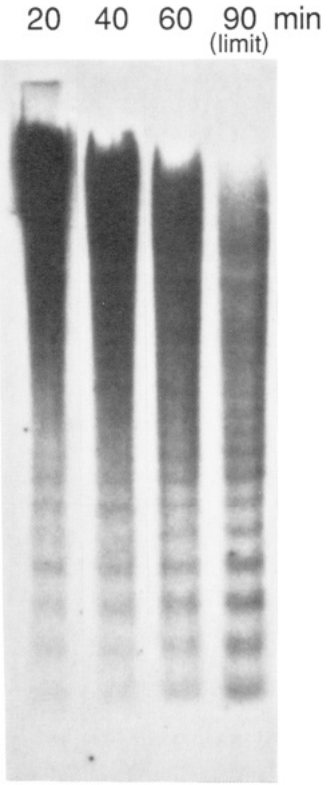

FIGURE 7: Time course of novanase digestion. Nuclei from 12-day erythroid cells were digested with novanase extract from adult erythrocytes for the times indicated and then electrophoresed, blotted, and hybridized to the "−10" probe.

Discussion

Nucleosome Spacing Is Compressed throughout the Active Chromatin Domains of Chick Erythroid Cells. Nucleosomes are more closely spaced in the active chromatin of both luxury genes (the ρ and β globins) and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase and the actins) in chick erythroid cells. At least in the case of the β-globin genes, the shortened repeat is a property not only of the genes themsleves but also of the entire active domain including the intergenic and the flanking regions beyond 10 kb upstream and 2 kb downstream. Moreover, this is true for erythroid cells of all developmental stages, but not for MSB cells, which are of nonerythroid origin. The physical domain and developmental regulation of compressed nucleosome spacing therefore parallel those of the regional DNase I sensitivity of active chromatin which also encompasses large domains and is developmentally regulated (Alevy et al., 1984; Jantzen et al., 1986; Lawson et al., 1982; Scott et al., 1987) and which also includes the complete region shown in Figure 1 (Lundell & Martinson, 1989; Stadler et al., 1980; Villeponteau et al., 1984; Lundell and Martinson, unpublished results).

Curiously, the regions at −10 and +2, which flank the domain of compressed nucleosome spacing, exhibit a degree of compression essentially the same as each other but reproducibly different from that of the other active region probes (summarized in Figure 9). Moreover, unlike the results for the other probes, nucleosome spacing for the −10 probe (and possibly also the +2 probe) appears to remain constant for all erythroid developmental stages. The −10 and +1 regions do not cross-hybridize. It will be interesting to determine whether they share other properties which may characterize domain-flanking chromatin. The probe at +6 (see Figure 1) gives nucleosomes spacing values approaching that of the inactive probes (Figure 9), signaling, perhaps, the end of the domain.

Different but Well-Defined Spacing Periodicities Coexist in Active Chromatin. The cleavage of 12-day erythroid chromatin in situ shows clearly that the spacing of nucleosomes over active chromatin in erythroid cells cannot be represented

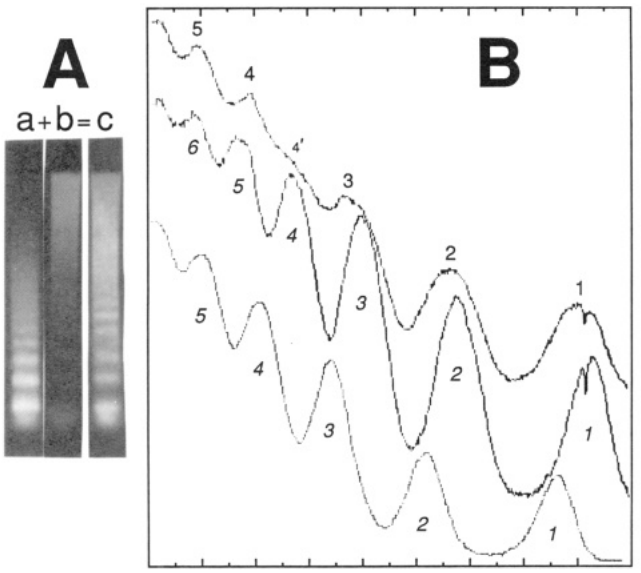

FIGURE 8: Reconstruction experiments showing the addition of two ladders of differing periodicity. (A) Lane a, yeast chromatin ladder with a 165 bp repeat; lane b, erythroid cell chromatin ladder with a 195 bp repeat; lane c, lanes a and b superimposed to give a composite pattern. (B) The top scan is a computer addition of the two lower scans, which are ladder patterns aligned to simulate superimposed 195 and 167 bp repeats in the composite.

in terms of a single series (Figure 6). There are two repeat lengths characteristic of erythroid active chromatin, one more compressed than the other, but both compressed relative to inactive chromatin.

Curiously, the more closely spaced series is always represented in the shorter oligonucleosomes while the less closely spaced series appears in the longer oligonucleosomes (e.g., Figure 6). This result superficially resembles an artifact in which nucleosomes slide together as digestion proceeds. However, in our experiments, neither bulk chromatin nor inactive chromatin exhibited any sliding. Moreover, sliding would not be expected to produce two *discrete* series of regularly spaced nucleosomes. Finally, the time course shown in Figure 7, and others like it (not shown), argues further against this possibility: without exception, the nucleosome spacing at early times of digestion is, if anything, slightly shorter than that at late times. While we cannot rule out completely the occurrence of rapid, cooperative, cleavage-induced sliding confined to active chromatin (see below), it seems more probable that both spacing modes exist in native chromatin, with the chromatin of shorter spacing being more rapidly digested than the chromatin of longer spacing as suggested by the results of Figure 2. Accordingly, at early times of digestion, the more rapidly digested, closely spaced nucleosomes would, if anything, contribute more to the overall pattern than at later times.

In Figure 8, we have reconstructed, in two different ways, the results to be expected on the basis of such a model. In part A, we overlapped the negatives of two simple ethidium bromide ladder patterns representing different nucleosome spacings and different degrees of digestion (lanes a and b) to obtain a composite pattern (lane c). In part B, we show the computer summation (top trace) of two gel scans aligned so as to simulate different nucleosome periodicities. Both com-

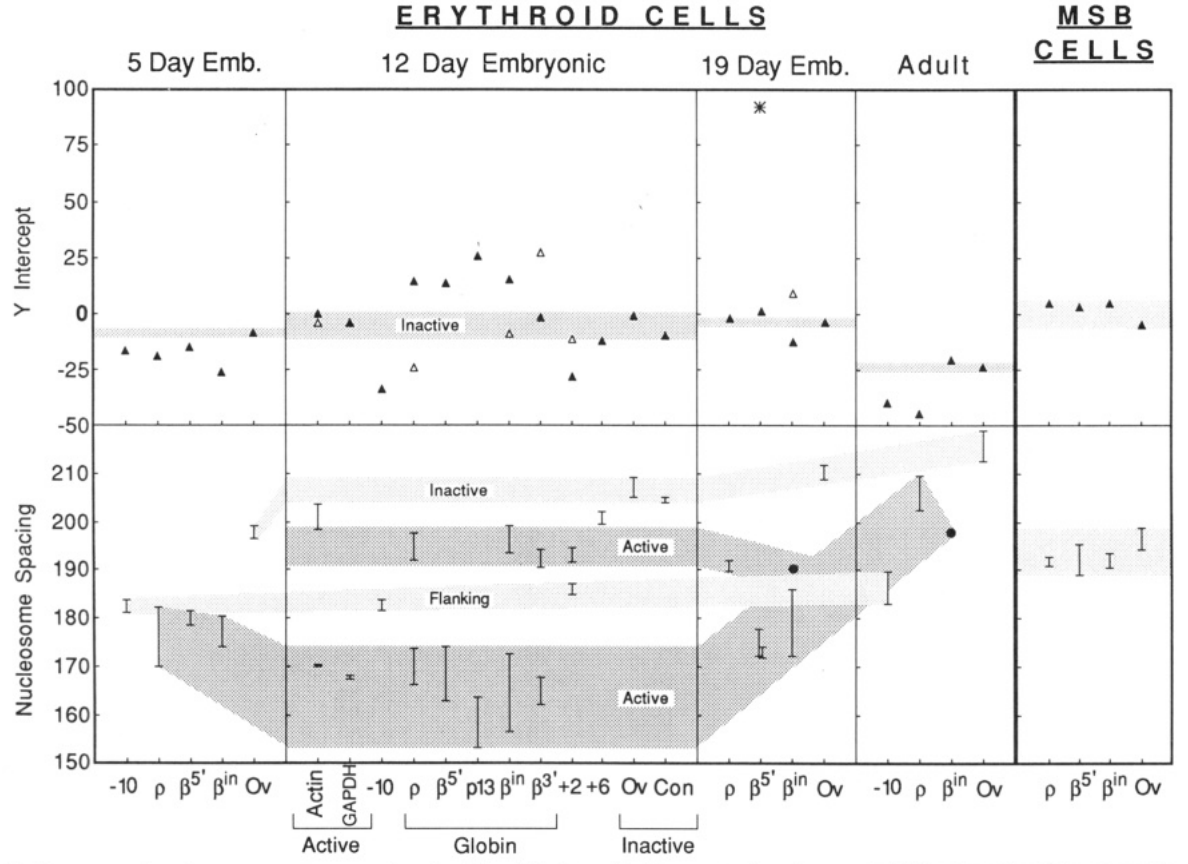

FIGURE 9: Summary of nucleosome spacing experiments. The data from all of our experiments were pooled and analyzed by regression analysis. In the lower set of panels, the error bars correspond to $\pm s_b$, the standard error of the slope (Mansfield, 1983). Data points without error bars are shown for instances in which the slope is based only on two bands in the gel. In the upper set of panels, the open triangles are for the intercepts of the upper regression lines in cases where the data yield two lines of different slope as in Figure 6. However, note that the specific values given for the individual experiments of Figures 4 and 6 may differ from the summary values of Figure 9 which represent pooled data. The star in the 19-day panel corresponds to the large positive $\beta^{5'}$ intercept of Figure 4C.

posites are very similar to our experimental results (compare Figure 8A, lane c, with the β^{intron} lane of Figure 2C′ or with lane 2 of Figure 5; compare the top trace of Figure 8B with the β^{intron} scans of Figure 3B,C) and support the interpretation that the two different series represent two different states of active chromatin which are digested by novanase to different extents. These two states may be interconvertible in chromatin, or their presence may reflect cell heterogeneity.

Although our results are clear concerning the existence of the two different nucleosome periodicities in active chromatin, their appearance from experiment to experiment is somewhat variable. In some experiments such as those for Figure 6, a doublet is clearly visible where the shorter and the longer periodicities overlap. Rarely, a ladder of doublets can be seen when the overlap is more extensive (e.g., ρ lane in the experiment of Figure 2E). Often, however, evidence for one series or the other is absent from the data. For example, in panel B of Figure 2, the ρ lane shows only the longer series, but in panel E, both series are evident. This variability, combined with the use of micrococcal nuclease, may explain why compressed nucleosome spacing in erythroid active chromatin has not been noticed in previous studies.

The results of all of the experiments described so far, as well as the results of many additional experiments, are summarized in Figure 9. For the definitive erythroid cells of 12-day embryos, the nucleosome spacing values for active chromatin fall into two clearly delineated ranges of values (darker shading) which correspond, for example, to the upper and lower series of Figure 6A,B. The exception, actin, "proves" the rule. The various actin genes are differentially regulated and tissue-specific (Ruzicka & Schwartz, 1988), so cross-hybridization of the β-actin probe presumably includes both active and inactive members of the actin gene family in erythroid cells. As a result, the upper series for actin falls between the ranges for active and inactive in Figure 9.

The distinction between the upper and lower ranges of active nucleosome periodicities diminishes for the definitive erythroid cells of 19-day embryos. The primitive erythroid cells of 5-day embryos may lack two distinct active nucleosome periodicities.

Relation of These Studies to Previous Reports. Sun et al. (1986) have studied the digestion characteristics of β-globin chromatin in adult chicken erythrocytes using micrococcal nuclease. Micrococcal nuclease digests active chromatin more rapidly than inactive chromatin (Wood & Felsenfeld, 1982) with the result that the trimming activity intrinsic to this enzyme is more pronounced for active oligonucleosomes than for inactive ones (Smith et al., 1983). For mature chicken erythrocytes, this exonucleolytic trimming of active oligonucleosomes by micrococcal nuclease can shift the oligonucleosome patterns dramatically. Differences in nucleosome periodicity can therefore be overlooked, and concluded to be absent (Sun et al., 1986), without a graphical analysis of the data. In Figure 10, we have plotted some of the data from Figure 4 of Sun et al. (1986) and find that their results agree well with ours. First, the spacing for active nucleosomes is less than for inactive. Second, the difference in spacing between active and inactive is diminished, but persists even in mature erythrocytes. Third, exonucleolytic trimming is more extensive in mature erythrocytes than in younger cells (see y interceps in Figures 9 and 10). All of the other data in Sun et al. (1986), when plotted (not shown), also agree with the data we have presented here.

Widmer et al. (1987) have studied the chromatin structure of the Balbiani ring region of a Chironomous cell line. This region is inactive in this cell line and is digested by micrococcal

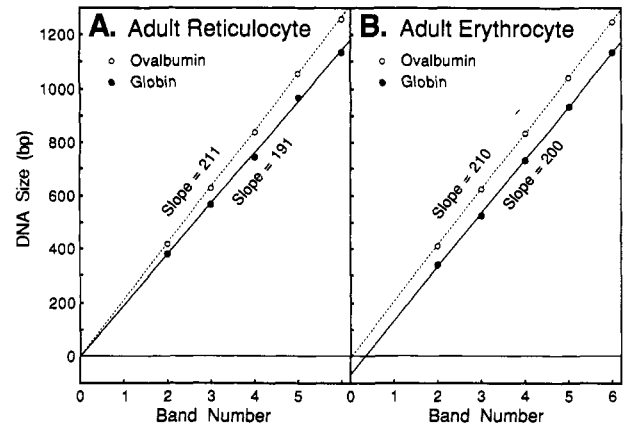

FIGURE 10: Linear regression analysis of data from Figure 4 of Sun et al. (1986).

nuclease even more slowly than bulk chromatin (Widmer et al., 1987). Thus, it resembles the ovalbumin chromatin in our studies. However, Widmer et al. (1987) found that micrococcal nuclease digests of this inactive chromatin yielded two spacing series in the gel, in superficial resemblance to the bimodality of our results for active chromatin. Their result is difficult to interpret, however, because the spacing of bands for the upper series in their gels is, in all cases, identical to the spacing of an underlying sequence repeat which exists in the DNA of the region. Thus, their result could conceivably be the consequence of a nucleosome ladder overlapping a DNA repeat ladder. Widmer et al. (1987) consider this explanation unlikely, largely because they find, as we do, a relatively narrow transition zone between the two different periodicities in the gel. However, we have shown in Figure 8 that the simple addition of two ladders can give a surprisingly narrow transition zone. Therefore, at the present time, we prefer the interpretation that their gel patterns arise from overlapping DNA repeat and nucleosome spacing ladders rather than from two ladders reflecting different nucleosome spacings.

Perhaps the first study to address nucleosome spacing in active polymerase II chromatin was by Gottesfeld and Melton (1978). They concluded that the spacing in active chromatin is the same as for bulk. However, they used a cDNA probe prepared from bulk mRNA. This probe certainly contained repetitive sequences, and hybridization to inactive domains probably dominated the pattern.

Smith et al. (1983) have also studied nucleosome spacing in active and in inactive chromatin and have found, as we have, that individual gene regions possess characteristic nucleosome spacing values which vary according to whether the genes are active or inactive. This supports the conclusion that nucleosome periodicity is a developmentally controlled property of chromatin. However, in contrast to our results, which show that nucleosome spacing in chick erythroid active chromatin is compressed, Smith et al. (1983) reported that nucleosome spacing in mouse cell active chromatin is expanded. Assuming the validity of their analysis (the scans in Figure 2 of Smith et al. do not agree with the rest of their data), this indicates that compression of nucleosome spacing is not a universal feature of active chromatin. Nevertheless, the results of Smith et al. (1983) support our general conclusion that the nucleosome repeat pattern of active chromatin is not subject to the same constraints as that of inactive chromatin.

Nucleosomes of Active Chromatin Are Mobile. The simplest interpretation of our results is that the nucleosomes of active chromatin, being less constrained by linker histone binding (Ridsdale et al., 1988; Weintraub, 1984), are free to

move closer together, perhaps following a critical replication event during development. It is well documented, in vitro, that lack of linker histones can lead to more closely spaced nucleosomes (Rodriguez-Campos et al., 1989; Stein & Bina, 1984; Watkins & Smerdon, 1985). Altered linker histones in active chromatin, which have been shown to bind with less affinity (Ridsdale et al., 1988 Weintraub, 1984), may yield a similar result. This is also the interpretation favored by D'Anna and Tobey (1989), who have reported results comparable to ours for chromatin domains involved in replication (i.e., replicons). They found that during the cell cycle in Chinese hamster cells the nucleosome repeat length decreases shortly before replication. Inasmuch as the decrease in repeat length is not a *consequence* of replication, this phenomenon resembles our observation that the globin domain repeat length is decreased in both nontranscribed and transcribed regions.

As mentioned earlier, our data do not establish definitively that, in vivo, the nucleosomes of active chromatin are more closely spaced. The nucleosomes may respond in some novel way to mild chromatin cleavage by moving rapidly and cooperatively closer together in vitro. For example, Glotov et al. (1982) have reported a transient cleavage-induced, topology-dependent shuffling of nucleosomes in SV40. Perhaps the global change in chromatin topology which accompanies the early stages of chromatin cleavage in nuclei (Hyde, 1982) triggers nucleosome movement to an extent which may be negligible in inactive domains but substantial in active domains. Such an effect may vary depending on the cell type. Thus, in mouse cells, the nucleosomes of active chromatin may slide further apart rather than closer together (Smith et al., 1983). Incomplete (albeit quantized) sliding also could explain the presence of two superimposed spacing series for a single probe in our results (e.g., Figure 6) as well as the fact that occasionally one or the other of the series is not observed. However, whether the movement occurs in vivo or in vitro, and whether movement is to longer or to shorter repeat lengths, the nucleosomes of active chromatin, but not of inactive chromatin, must be modified by some domain-wide influence that enhances their mobility throughout the domain.

Smith, P. A., et al. (1984) have invoked a special sliding response of this type to account for their observation that nucleosome spacing in newly replicated chromatin appeared to be shorter than the average unless cross-linking was carried out prior to analysis. However, they did not determine what aspect of their procedure incurred the sliding. If it was during the nuclear isolation step or during handling of the isolated nuclei [steps which have been shown to alter chromatin structure (Zhang & Gralla, 1990)], then their conclusions are not applicable to our close-spacing results which were obtained even in situ with no nuclear isolation step. We have attempted to use their whole-cell cross-linking procedure to test for sliding in our system but have not been successful in obtaining adequately resolved nucleosomal ladders in our gels following cross-link reversal.

In conclusion, we wish to emphasize that any preferential movement of the nucleosomes of active chromatin following cleavage in our experiments would be a phenomenon qualitatively different from the usual type of in vitro sliding which has been so extensively reported in the literature. First, all studies of nucleosome sliding so far reported have involved bulk chromatin [e.g., see Watkins and Smerdon (1985)]. The close spacing we report here involves only active chromatin, and is documented for conditions under which no sliding of nucleosomes occurs in either bulk chromatin or the chromatin of specific inactive genes. Second, nucleosome sliding for bulk chromatin either is very slow (Beard, 1978; Jack & Eggert, 1990; Jackson et al., 1990; Sidik & Smerdon, 1990), is slight (Glotov et al., 1982), or is detected at significant levels only after extensive nuclease digestion (Watkins & Smerdon, 1985; Weischet & Van Holde, 1980). In contrast, if the close spacing we report arises during the analysis, it must do so early (Figure 7), it must be cooperative and extensive in order to yield the long evenly spaced arrays illustrated in Figure 4, and it must be a direct consequence of mild cleavage per se, since it occurs even in situ within the whole cell with no intervening nuclear isolation step (Figure 5). Moreover, trimming of the oligonucleosomes following the sliding would need to be very rapid and efficient in order to yield the discretely shorter DNA lengths characteristic of the more closely spaced series.

Perhaps a signature property of nucleosomes in active chromatin is that they are designed to be mobile. Thus, nucleosome mobility, even in vitro, may be a direct reflection of in vivo function.

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