Gamma Rays and Bleomycin Nick DNA and Reverse the DNase I Sensitivity of β -Globin Gene Chromatin In Vivo

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The active β -globin genes in chicken erythrocytes, like all active genes, reside in large chromatin domains which are preferentially sensitive to digestion by DNase I. We have recently proposed that the special structure of chromatin in active domains is maintained by torsional stress in the DNA (Villeponteau et al., Cell 39:469-478, 1984). This hypothesis predicts that nicking of the DNA within any such chromosomal domain in vivo will relax the DNA and lead to loss of the special DNase I-sensitive state. Here we have tested this prediction by using gamma irradiation and bleomycin treatment to cleave DNA within intact chicken embryo erythrocytes. Both treatments cause reversal of DNase I sensitivity. Moreover, reversal occurs at approximately one nick per 150 kilobase pairs for both agents despite their entirely unrelated modes of cell penetration and DNA attack. These results suggest that the domain of DNase I sensitivity surrounding the β -globin genes comprises 150 kilobase pairs of chromatin under torsional stress and that a single DNA nick in this region is sufficient to reverse the DNase I sensitivity throughout the entire domain.

As first discovered by Weintraub and Groudine (44, 50), active genes in higher eucaryotes reside in chromosomal domains which are more sensitive to digestion by DNase I than the surrounding inactive chromatin (reviewed in reference 30). For most genes these domains extend well beyond the limits of actual transcription (30). In the three cases for which the domains have now been completely mapped they have been found to encompass 12 kilobases for the chicken glyceraldehyde-3-phosphate dehydrogenase gene (1), 24 kilobases for the chicken lysozyme gene (19), and 100 kilobases for the ovalbumin gene family (24).

Two questions are paramount concerning the nature of these DNase I-sensitive domains. First, of course, is the question of the nature of the structural modifications which render these domains more susceptible than bulk chromatin to digestion by DNase I. The second question, however, is more intriguing, namely, how are these domain-specific structural modifications imposed so uniformly over such enormous chromatin fiber distances? Any model for the establishment of "active" chromatin during differentiation must account for the propagation of this structural alteration along the chromatin throughout domains which may encompass hundreds of nucleosomes. It is useful to recognize that although this problem is superficially similar to that of "action at a distance" (as for enhancer sequences), it differs in that the effect is not targeted to a specific position (such as one or more promoters) but rather alters structure uniformly throughout a domain. It is therefore more properly "action through a distance."

In considering what strategy a cell might use to alter chromatin structure throughout large domains we recognize that the fundamental problem is not so much to find a solution to the structure of active chromatin but rather to find an answer to the question of how the instructions to adopt a new conformation are transmitted with such unifor-

mity through such great distances. Among the conceivable strategies for the dissemination of information throughout a chromatin domain, two alternatives appear to be more likely than others. On the one hand the particular structure for chromatin may be specified at the initiation of replication and then laid down processively as the replication fork advances (45, 48). Alternatively, for any topologically independent chromatin domain, the degree of torsional stress in the DNA may specify the type of structure to be assembled or maintained (3, 27, 28, 32, 40, 46). These possibilities are not mutually exclusive.

A correlation between domains of function and the organization of nuclear DNA into domain-sized loops has frequently been noted (24, 30, 48). The existence of DNA loops, securely tethered at either end to a nuclear matrix, provides a simple physical rationale for the existence of large, yet discrete, functional domains in chromatin. Moreover at least some of these domains, at least in higher eucaryotes, appear to contain unrestrained supercoils (torsional stress) in vivo (3, 27, 30, 32). However, the molecular mechanism by which the cell generates this torsional stress is not yet understood (9, 16, 21, 46).

We have previously presented data consistent with the hypothesis that torsional stress is required for the maintenance of the DNase I-sensitive state of active chromatin in vivo (40). We reported that treatment of intact chicken erythrocytes (RBCs) with novobiocin reverses the DNase I-sensitive state of the β -globin genes such that they come to resemble inactive chromatin (40). This reversal is not a simple cell killing effect, since many other cytotoxic drugs have no effect on preferential DNase I digestion of the active β -globin genes (40). Based on the fact that novobiocin is a topoisomerase II inhibitor, we suggested that the DNase I-sensitive conformation of active chromatin might be induced and maintained by torsional stress. We also showed, consistent with this conclusion, that cleavage of nuclei with micrococcal nuclease in vitro also leads to a loss of the DNase I-sensitive conformation of the active globin genes.

The torsional stress hypothesis for DNase I sensitivity predicts that DNA integrity must be maintained throughout

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the entire domain *in vivo* if torsional stress and, in turn, DNase I sensitivity are to be maintained. Moreover, the hypothesis predicts further that the domains of DNase I sensitivity should be susceptible to target size analysis based on DNA nicking *in vivo* and that the resulting apparent target sizes should be independent of the nature of the nicking agent.

To test the torsional stress hypothesis, we have now introduced DNA nicks into the genome of intact RBCs. To generate *in vivo* nicking of the genomic DNA we have exposed embryonic chicken RBCs either to gamma irradiation from a cobalt source or to the chemical DNA-cleaving agent bleomycin. Both treatments reverse the DNase I sensitivity of the β -globin gene region as predicted by the torsional stress model. Moreover, reversal of the DNase I sensitivity of the active β -globin gene occurs at the same nicking frequency (about 1 nick per 150 kilobase pairs) for both methods of DNA cleavage despite their entirely unrelated modes of attack.

MATERIALS AND METHODS

Gamma irradiation. Fertilized White Leghorn chicken eggs were incubated for 12 days in a Humidaire model 50 incubator (Humidaire, New Madison, Ohio). Blood was collected by vein puncture and dispensed into cold 0.14 M NaCl–2.7 mM KCl–1.5 mM KH_2PO_4 –8 mM Na_2HPO_4 (phosphate-buffered saline). RBCs were pelleted free of serum and suspended in phosphate-buffered saline at a concentration of 4×10^6 cells per ml. Samples of this cell suspension were irradiated in individual 15-ml plastic tubes (Corning Glass Works, Corning, N.Y.) in an ice bucket. To keep DNA strand scission linear with radiation dose, all RBC samples were irradiated on ice at the same radius (13 cm) from the ^{60}Co source (flux, 5.7 kilorads per min), and only the time of irradiation was varied. Repair does not occur at 0°C (18).

For many of our experiments the RBC samples were incubated for 20 min at 37°C after gamma irradiation and before nuclear isolation. We are unaware of any studies which have looked at DNA repair in postmitotic 12-day chicken RBCs, but we expect it to be low or absent. In control experiments we were not able to detect any increase in DNA size during 20 min at 37°C for 12-day RBCs previously exposed to 50 kilorads of gamma irradiation (data not shown). We conclude that significant repair of DNA nicks in irradiated 12-day RBCs does not occur during the 37°C incubation of our experiments.

We have found that the proportion of DNA cleaved decreases as the cell concentration at a given irradiation dose increases. Perhaps gamma irradiation generates long-lasting free radicals or other reactive species in the buffer which are depleted to a greater extent by larger numbers of cells, lessening the degree of damage to individual cells (18). Thus, for example, at an RBC concentration of 10^8 cells per ml, doses severalfold higher than those we have reported in this paper are required to nick the DNA to the same extent (data not shown). However, reversal of DNase I sensitivity is always correlated with the extent of DNA cleavage and not with the particular dose of gamma irradiation used (data not shown).

Bleomycin treatment. RBCs were pelleted and then suspended in small volumes of phosphate-buffered saline. The cells were prewarmed at 37°C for 5 min, made 0.2 mM in dibucaine, and incubated for 15 min at 37°C. The dibucaine-treated cells were then made 100 or 200 μM in bleomycin and incubated for 20 to 60 min at 37°C before cell pelleting and lysing.

Isolation of nuclei. RBCs were pelleted at $1,500 \times g$ for 3 min in a table-top centrifuge and lysed by suspending in at least 40 volumes of 10 mM Tris hydrochloride (pH 7.4)–10 mM NaCl–3 mM MgCl_2 –30% glycerol–0.3 M sucrose (RSBgs buffer) and 0.4% Nonidet P-40. After 1 min on ice the nuclei were pelleted at $2,000 \times g$ for 5 min and suspended in a small volume of RSBgs buffer without Nonidet P-40. The final DNA concentration was normally adjusted to 500 $\mu\text{g}/\text{ml}$ before DNase I digestion.

Vertical alkaline agarose gel electrophoresis. Hot 0.7% agarose dissolved in water was cooled to 80°C, made 30 mM in NaOH and 1 mM in EDTA, and then poured into a vertical slab mold. After cooling to room temperature, the gel was mounted and further cooled to 0°C in electrophoresis buffer (30 mM NaOH and 1 mM EDTA).

The DNA samples were made 50 mM in NaOH, 1 mM in EDTA, and 3% in Ficoll with marker dyes. The DNA was then denatured at 37°C for 20 min, loaded onto the cold gels, and electrophoresed for 60 to 90 min at 0°C. After electrophoresis, the gels were neutralized with 0.1 M Tris hydrochloride (pH 7.0) and stained with ethidium bromide to visualize the DNA.

Nuclease digestion and DNA isolation. Nuclei in 400 μl of RSBgs buffer were prewarmed to 37°C and then digested with 8 μg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml for 3 min, which digests about 10% of the DNA to acid solubility. All digestions were stopped by making the sample 0.2% in sodium dodecyl sulfate and 25 mM in EDTA. The surviving DNA was then made 50 $\mu\text{g}/\text{ml}$ in protease K, incubated for 16 h at 37°C, and extracted several times with phenol-chloroform (1:1). The DNA was ether extracted twice, treated with 100 μg of RNase A per ml at 37°C for 2 h, and ethanol precipitated.

Other methods. Dot blotting and blot hybridization were as described previously (40). Autoradiographs of the dot blots were scanned with a Quick Scan densitometer. As a control, all labeled probes were hybridized to Southern blots (37) of restricted genomic chicken DNA to check the ratio of signal to background.

We used several controls to monitor preferential DNase I digestion. Staphylococcal nuclease does not preferentially digest the globin genes (50), so we used DNA from staphylococcal nuclease-digested 12-day RBC nuclei to calibrate the hybridization to the DNase I-digested samples. As a probe for inactive genes we used a chicken ovalbumin clone (from B. O'Malley). Hybridization of the DNase I-digested samples to the inactive probe was used to calibrate the 100% level that an active gene should reach on complete reversal to the inactive conformation.

RESULTS

Gamma irradiation reverses the preferential DNase I sensitivity of the active β -globin gene domain. All of our analyses in this paper were carried out on 12-day embryonic RBCs, which express primarily the adult β -globin genes (17, 23, 39). Previous studies have shown that DNase I preferentially digests the β -globin genes in embryonic RBC nuclei but not nuclei of other cell types (40, 50).

If it is torsional stress which maintains the DNase I-sensitive state in the β -globin domain, then nicking the DNA *in vivo* by gamma irradiation of whole cells should both relax torsional stress and induce a reversal of preferential DNase I sensitivity. To test this prediction, we gamma irradiated 12-day RBCs with 12.5 to 100 kilorads at 0°C with a radioactive ^{60}Co source. After 20 min of incubation at 37°C,

FIG. 1. Hybridization probes for the chicken β -globin domain (39). Probe 1 is from the β^H -globin gene but cross-hybridizes to the other β -globin genes as well. Probe 2 is specific for the intergenic DNA between the β^H - and β -globin genes.

the cells from each radiation dose were pelleted and lysed, and the nuclei were isolated. Isolated nuclei from various cell samples were digested with pancreatic DNase I until 10 to 15% of the DNA was rendered acid soluble. The remaining DNA was then spotted onto nitrocellulose and hybridized to globin gene probes G1 and G2 (Fig. 1) or to an ovalbumin gene probe. Figure 2 shows the resulting dot blots. Hybridization intensities of the dot blots were quantitated by densitometer scanning and expressed as a percentage of ovalbumin probe hybridization (Fig. 3).

Irradiation of RBCs induced a reversal of the preferential DNase I sensitivity of the globin genes (Fig. 2 and 3). As for novobiocin treatment (40), the reversible component of DNase I sensitivity accounted for about 40% of the total. This plateau was reached at 25 kilorads of irradiation. These results confirm the prediction of the torsional stress hypothesis and suggest that 25 kilorads of gamma irradiation introduces sufficient nicking within the β -globin domain to relax torsional stress and reverse the preferential DNase I sensitivity.

The antitumor drug bleomycin reverses preferential DNase I sensitivity. If DNA nicking relieves torsional stress and leads to reversal of DNase I sensitivity as we suggest, then any agent which introduces nicks into the genome in vivo as does gamma irradiation should relieve torsional stress and

FIG. 2. Gamma irradiation of RBCs induces reversal of DNase I sensitivity. Twelve-day embryonic RBCs were gamma irradiated with 0, 12.5, 25, 50, or 100 kilorads at 0°C and then incubated at 37°C for 20 min. The nuclei were isolated and digested with DNase I. The isolated DNA from all of the samples was spotted in three sets of duplicate dots onto nitrocellulose. The blots were then hybridized to globin probe G1 or G2 (Fig. 1) or to a probe for the inactive ovalbumin gene.

FIG. 3. The dot blot autoradiographs in Fig. 2 were scanned. In preparing the histograms all hybridization data for DNase I-treated nuclei were first normalized to the hybridization obtained for total genomic DNA (from staphylococcal nuclease-treated nuclei) and then expressed as a percentage of ovalbumin hybridization to identical dots.

reverse the DNase I sensitivity of active genes. The antitumor drug bleomycin is known to cleave chromosomal DNA in vivo as well as purified DNA in vitro (6). Bleomycin is a low-molecular-weight glycopeptide which can readily cross the plasmid membrane of intact cells when the cells are pretreated with low concentrations of the local anesthetic dibucaine to enhance membrane fluidity (6).

To determine whether bleomycin treatment of RBCs can lead to reversal of DNase I sensitivity, we preincubated 12-day RBCs with dibucaine for 15 min and then added 100 μ M bleomycin for 20, 40, or 60 min of incubation (Fig. 4, bars 2 through 4) or 200 μ M bleomycin for 60 min (bar 5). Bar 1 is a control sample incubated for 75 min in dibucaine

FIG. 4. Treatment of RBCs with bleomycin reverses preferential DNase I sensitivity. Dibucaine-treated cells were incubated for 20, 40, and 60 min (bars 2, 3, and 4) in 100 μ M bleomycin or for 60 min without bleomycin (control, bar 1) or with 200 μ M bleomycin (bar 5). The nuclei from these samples were then isolated and assayed for preferential DNase I sensitivity.

FIG. 5. Single-stranded DNA size after whole cell gamma irradiation. DNA from the gamma-irradiated RBC samples of Fig. 3 was isolated from the nuclei before DNase I digestion, denatured at 37°C for 20 min in 50 mM NaOH, and then electrophoresed on a horizontal alkaline 1% agarose gel (A) or on a high-resolution vertical alkaline 0.7% agarose gel (C). The gels in A and C were visualized by ethidium bromide staining. (B) Southern blot autoradiograph of the gel in A (hybridized to probe G2). The DNA samples are RBCs gamma irradiated with 0, 25, 50, and 100 kilorads (lanes 1, 2, 3, and 4, respectively); *Hind*III-restricted lambda (lane 5); uncut lambda (lane 6); nonirradiated control (lane 7; same as lane 1).

without bleomycin and shows that dibucaine has little or no effect by itself on DNase I sensitivity. The data in Fig. 4 clearly demonstrate that increases in bleomycin concentration or time of exposure lead to increased levels of reversal of preferential DNase I sensitivity. With 200 μ M bleomycin for 60 min, RBCs are reversed to an extent similar to that of RBCs treated with novobiocin for 60 min (40) or gamma irradiated at 25 kilorads (Fig. 2 and 3).

Taken together these data provide strong support for the hypothesis that torsional stress is required to maintain the DNase I-sensitive state of active genes.

Estimation of the single-stranded DNA sizes after nicking with gamma irradiation or bleomycin. To obtain an estimate of the average size of the torsionally stressed domain which encompasses the globin genes, we examined the extent of DNA nicking which accompanies the loss of DNase I sensitivity. For this purpose we withdrew samples of DNA from the gamma-irradiated cells of Fig. 2 and 3 before the DNase I digestion step, and analyzed it by alkaline agarose gel electrophoresis (Fig. 5). A detectable decrease in single-strand DNA length occurred as the irradiation dose was increased from 0 (Fig. 5A, lane 1) to 25, 50, and 100 kilorads

(lanes 2, 3, and 4, respectively). Figure 5B shows a Southern blot of this same gel hybridized to a globin probe. We have carefully compared the original autoradiogram shown in Fig. 5B with the original photograph of the ethidium bromide-stained gel shown in Fig. 5A; we have concluded that the DNA in the globin region is nicked to the same average size as the bulk DNA. This confirms, as expected, that gamma irradiation causes nicking at random throughout the genome.

To obtain a size estimate for the DNA of the irradiated samples we compared the DNA isolated from the gamma irradiated cells with a lambda DNA standard by using a higher-resolution gel. Figure 5C shows the migration of the same samples exposed to 0, 25, 50, and 100 kilorads (lanes 1 through 4, respectively) compared to intact lambda DNA (lane 6). Despite the poor resolution in this molecular weight range, it is clear that the samples exposed to 0, 25, and 50 kilorads (lanes 1 through 3) are all of larger size than the lambda DNA (lane 6). Only the sample of RBC DNA that was exposed to 100 kilorads (lane 4) is comparable to lambda DNA in size. From Fig. 5C and similar gels we estimate that the size of the sample exposed to 100 kilorads is actually slightly less than that of lambda DNA (i.e., ca. 30 to 50 kilobases).

We wish to estimate the size of the DNA from the 25 kilorad-irradiated cells since 25 kilorads is the minimum dose which gives complete reversal (Fig. 2 and 3). Although these gels cannot provide us with meaningful size estimates for such high-molecular-weight DNA, we can nevertheless estimate approximate DNA sizes from the linear relationship between the dose and extent of DNA nicking for gamma irradiation (36). Thus the DNA from 25 kilorad-irradiated cells is probably about four times the molecular weight of the 100-kilorad DNA, or about 160 kilobases. Therefore one nick per 160 kilobases is sufficient to lead to maximal gamma-induced reversal of DNase I sensitivity (Fig. 3A and B).

We assume that a single nick in either strand is sufficient to relieve torsional stress in a domain (5, 8, 36). Therefore one nick per 160 kilobases of a single strand implies one nick per 80 kilobase pairs of double-stranded DNA at 25 kilorads of irradiation. Since reversal is maximal at 25 kilorads, we assume that all domains have been nicked, meaning that the average number of nicks per globin domain at 25 kilorads is >1 . Therefore, since at 25 kilorads there is one nick per 80 kilobase pairs but more than one nick per domain, the domain size for the β -globin gene region must be larger than 80 kilobase pairs.

Conversely at 12.5 kilorads reversal is half maximal, so we assume that the average number of nicks per domain at this dose is <1 . Since the number of nicks at 12.5 kilorads is presumably half that at 25 kilorads, this suggests an upper limit for the domain size of 2×80 , or 160 kilobase pairs. Thus based on gamma irradiation we estimate the functional domain size for the chicken β -globin gene region to lie in the range of 80 to 160 kilobase pairs. Since we have measured single-strand DNA lengths in alkali, which overestimates somewhat the actual number of cuts in vivo (18), this is actually a modest underestimate of the true β -globin gene domain size. For simplicity we will henceforth refer to this estimate of domain size as being 150 kilobase pairs.

We have also examined the extent of DNA nicking which accompanies the loss of DNase I sensitivity caused by bleomycin. Selected DNA samples from the experiments presented in Fig. 3 and 4 were electrophoresed on alkaline gels as described for Fig. 5C. DNA samples from RBCs treated for 60 min with 0, 100, or 200 μ M bleomycin (bars 1,

FIG. 6. Single-stranded DNA size of the β -globin region following bleomycin treatment. DNA from the bleomycin-treated RBC samples used in Fig. 4 or the 25 kilorad-irradiated sample used in Fig. 3 were isolated from nuclei before DNase I digestion, denatured at 37°C for 20 min in 50 mM NaOH, electrophoresed on a high-resolution vertical alkaline 0.7% agarose gel, and then Southern blotted to nitrocellulose. (A) Ethidium bromide stained gel. (B) Autoradiogram of Southern blot hybridized to globin probe G2. DNA samples: intact lambda DNA (lane M); nontreated control RBCs (lane 1); RBCs treated with 100 (lane 2) or 200 (lane 3) μ M bleomycin for 60 min; 25-kilorad gamma-irradiated RBC sample (lane 4).

4, and 5, respectively, in Fig. 4) are displayed in lanes 1, 2, and 3, respectively in Fig. 6. Lane 4 contains a DNA sample from RBCs which received 25 kilorads of gamma irradiation (same sample as shown in lane 2 of Fig. 5C).

Little or no change in bulk DNA size can be detected for these concentrations of bleomycin (lanes 1 through 3 of Fig. 6A), consistent with the results of others (6). However, bleomycin has a 10-fold preference for nicking within active gene regions (22). Therefore, the gel in Fig. 6A was Southern blotted and hybridized to globin probe G2. The autoradiogram of Fig. 6B confirms that nicking can be detected in the β -globin domain (lanes 1 through 3). Comparison of gel mobility with respect to full-length lambda DNA (lane M) and 25-kilorad gamma-irradiated RBC DNA (lane 4) shows that reversal for bleomycin occurs at a similar extent of DNA nicking as for gamma irradiation. (Recall that for gamma-irradiated DNA, single strand lengths measured in alkali are slightly less than the true lengths *in vivo*.)

Reversal of DNase I sensitivity *in vivo* is a delicate process. The gamma-irradiated cells of Fig. 3 were irradiated on ice and then incubated for 20 min at 37°C before DNA purification. Curiously, however, if the 37°C incubation is omitted to a substantial extent only for the lowest irradiation dose of 12.5 kilorads (Fig. 7). These data suggest that gamma irradiation gives rise to opposing effects in chromatin. On the one hand nicking of the DNA facilitates relaxation of torsional stress (Fig. 7; 12.5 kilorads). On the other hand, at high doses gamma irradiation apparently leads to further, unrelated damage which interferes with relaxation of the chromatin (Fig. 7; 25, 50, and 100 kilorads). Thus, Fig. 3 (25, 50, and 100 krad) shows that incubation at 37°C for 20 min in some way allows the impaired reversal process to proceed to completion even after high doses of irradiation. It is not surprising that the process of reversal is impaired in cells which have been traumatized by high doses of irradiation. Isolated nuclei, which inevitably become damaged during

isolation and maintenance *in vitro*, also demonstrate impaired reversal (40).

DISCUSSION

Is there torsional stress in eucaryotic chromatin? Our results show that only treatments which are capable of leading to relaxation of torsional stress lead to the loss of DNase I sensitivity in active chromatin (see below). The simplest interpretation of these results is that it is torsional stress which maintains the DNase I-sensitive state of active chromatin. What independent evidence is there for the existence of torsional stress in eucaryotic chromatin?

Using DNA topoisomerase I-mediated relaxation as an assay, Luchnik et al. (28) reported in 1982 that a small minority of simian virus 40 minichromosomes possess a high density of unrestrained DNA supercoils. However, because of the small fraction of material involved, it was very difficult to be certain that these were of biological origin. The following year Weintraub (46) presented evidence for the existence of torsional stress in eucaryotes *in vivo*, based on the generation of altered S1 nuclease sensitivity in certain DNA sequences after transfection. Subsequently, Ryoji and Worcel (32) presented data consistent with the report of Luchnik et al. (28), and Luchnik et al. (2, 27), in more recent papers, presented experiments supporting the conclusions of Weintraub (46). Recently Barsoum and Berg (3) showed that all simian virus 40 minichromosomes possess at least a low level of torsional stress.

In contrast, Sinden et al. (35) have reported no detectable torsional stress in eucaryotic chromatin, based on gamma irradiation followed by a trimethylpsoralin binding assay. However, they pointed out that if torsional stress were limited only to active chromatin domains in eucaryotes then it would not have been detected in their experiments. Moreover Sinden et al. (35) did not report any measurements

FIG. 7. Gamma irradiation of RBCs without a 37°C postincubation. One half of each irradiated cell sample of Fig. 3 was processed for DNase I digestion without any postirradiation incubation at 37°C. The isolated DNA from the samples was analyzed as described in the text.

of actual extents of DNA cleavage in those experiments involving eucaryotic cells. The extents of cleavage we have measured after similar doses of irradiation are 1 order of magnitude less than what they estimated based on previous calibrations with *Escherichia coli*. Since the conditions of irradiation can affect the dose-response relationship for gamma irradiation (18) (Materials and Methods), it is possible that Sinden et al. had less cleavage in the eucaryotic DNA than they realized and that the test for torsional stress in eucaryotes was less rigorous than they assumed.

Recently, Saavedra and Huberman (33) reported an elegant genetic study in which they failed to detect torsional stress in *Saccharomyces cerevisiae* 2 μ m plasmid DNA in vivo. As the authors point out it is difficult to assess the extent to which the 2 μ m plasmid is representative of all eucaryotic chromatin. However, at least with regard to active chromatin and the role torsional stress may play in that regard, it is clear that *S. cerevisiae* is not typical. *S. cerevisiae* does not possess a separate DNase I-sensitive chromatin compartment (25), presumably because it lacks H1 (7), which is now widely regarded as being instrumental in maintaining the inactive state of repressed chromatin in vivo (47, 48).

If there is torsional stress in eucaryotic chromatin in vivo, how might it be generated? There is as yet no report of an isolated eucaryotic gyrase, and the reported requirement for a gyrase-like activity during chromatin assembly and transcription (15, 20, 32) has been questioned (9, 13, 16, 34). Nevertheless, torsional stress does seem to be generated by some mechanism in eucaryotes, at least under experimental conditions (21, 32). Perhaps mechanisms not involving a putative gyrase generate torsional stress in eucaryotic chromatin (3, 46). In any case, for the purpose of the remainder of this discussion we will assume that at least a portion of the genome in higher eucaryotes is under torsional stress. As discussed below and in the introduction, the torsional stress hypothesis for maintenance of active chromatin not only is the simplest interpretation of the present data but also provides a simple rationale for the regulation of chromatin structure over extensive domains.

One nick per 150 kilobase pairs reverses the DNase I sensitivity of the β -globin genes. We have shown that exposure of intact RBCs to either gamma irradiation or bleomycin treatment results in the loss of the preferential DNase I sensitivity of the active β -globin gene domain. Aside from novobiocin we have found no other agent that is capable of inducing active chromatin to revert to an inactivelike state of DNase I sensitivity (40). Thus, only agents that cut DNA can cause reversal. (Note that novobiocin can also lead to DNA cleavage; see below.)

Moreover, reversal appears to be related strictly to the number of cuts in the β -globin domain. This number is similar for both gamma rays and bleomycin despite their unrelated modes of attack. The disparity in the overall cleavage mechanisms of these two agents is illustrated by the strong preference for cleavage of active chromatin by bleomycin (22) (Fig. 6) but lack of any preference by gamma rays (Fig. 5). Because of the preference of bleomycin for the active domains, one nick per 150 kilobase pairs is achieved in the neighborhood of the globin genes at a level of overall nicking that is so low as to be undetectable (Fig. 6). Thus reversal is correlated with the number of nicks in the active domain and is not correlated at all with the overall level of DNA (or other nonspecific) damage.

The simplest interpretation of these results is that DNA cleavage in large, torsionally stressed domains of chromatin

leads to dissipation of this stress and hence a change in the structure of the associated chromatin. Considering the approximate nature of our present measurements, we regard the estimate of 150 kilobase pairs for the functional size of the β -globin domain as being consistent with previous estimates for the average size of topologically independent domains of *Drosophila melanogaster* cells (85 kilobase pairs [5]) and mammalian cells (90 kilobase pairs [42]).

Although other, more general interpretations of our data are not ruled out, they lack the simplicity of the torsional stress hypothesis and do not offer any obvious explanation for how chromatin structure may be manipulated in vivo over large, well-defined domains. One general possibility is that the loss of DNase I sensitivity may derive, directly or indirectly, from a cellular response to DNA damage. One such response is poly-ADP-ribosylation, which is activated in direct consequence of DNA cleavage (4). However, the ability of gamma irradiation to induce reversal at 0°C (i.e., without a 37°C postincubation; Fig. 7, 12.5 kilorads) argues against any enzymatic mechanism for driving the reversal and therefore renders all generalized cellular response mechanisms unlikely. The direct physical response of torsional relaxation thus remains the simplest possibility.

Although we report here that one nick per 150 kilobase pairs is sufficient to relax active chromatin in vivo, we previously found that a much higher cutting frequency was required for reversal when nuclei were digested with staphylococcal nuclease in vitro (40). This indicates either that reversal is an active physiological process which is lost upon nuclear isolation or that chromatin in nuclei, suspended in nonphysiological buffers in vitro, has diminished freedom of movement. In keeping with this discussion we prefer the latter, physical explanation. This phenomenon is reminiscent of the observation by Lyderson and Pettijohn (29) that bacterial nucleoides are efficiently unfolded in vivo by gamma irradiation but are not unfolded by gamma irradiation after isolation in vitro.

Novobiocin-induced reversal may also be a consequence of DNA cutting. We previously reported that novobiocin, but none of several additional drugs tested, induces the reversal of DNase I sensitivity (40). However, we have recently discovered that novobiocin treatment of RBCs leads to the release in vivo of an unusual endogenous nuclease which introduces double-stranded DNA cuts into the RBC chromosome over a period of hours (41). Although we previously showed that full reversal of DNase I sensitivity occurs before any detectable cutting in the 10 kilobase pairs immediately surrounding the β -globin genes (40), our present work shows that 1 order of magnitude less DNA cleavage than one cut per 10 kilobase pairs leads to reversal. Thus, it is possible that novobiocin-induced reversal of DNase I sensitivity is mediated by rare DNA cutting events, undetected in our previous work, rather than by the inhibition of topoisomerase II.

Of course, the torsional stress hypothesis for the generation and maintenance of DNase I sensitivity is not affected by this alternate possible interpretation for the pathway by which novobiocin induces reversal. However, it leaves unspecified which of various possible mechanisms may be responsible for generating the torsional stress of active chromatin.

Recently Cotten et al. (9, 34) showed that histones can be precipitated by novobiocin in vitro. They suggested (9) that nonspecific disruption of chromatin in vivo may account for the reversal of DNase I sensitivity. However, as we reported (40), incubation of either whole cell lysates or isolated nuclei

in vitro with novobiocin does not duplicate the reversal phenomenon. Moreover, novobiocin-treated cells contain nuclei with normal nucleosomal spacing which yield clear multinucleosome ladders on DNA gels (41). These nuclei also display distinct hypersensitive sites and exhibit preferential accessibility to active chromatin of a linker-directed endogenous nuclease (41). Therefore the novobiocin-mediated reversal of DNase I sensitivity in vivo does not appear to have a trivial explanation involving wholesale chromatin disruption.

Torsional stress and DNase I sensitivity. How might torsional stress predispose chromatin toward a DNase I-sensitive state? What feature of chromatin structure is recognized by DNase I as sensitive? Mere differences in accessibility to DNase I owing to differential packing of chromatin at the level of higher order structure are unlikely to be the major factor contributing to differential DNase I sensitivity. Active genes remain DNase I sensitive even in metaphase chromosomes (14). Moreover, active chromatin that has lost most of its preferential sensitivity to DNase I as a result of novobiocin treatment remains preferentially accessible to an endogenous linker directed nuclease (41). On the other hand subtle changes in DNA helix geometry can have a significant effect on susceptibility to DNase I cleavage (10–12, 26, 31). Therefore it is not necessary to imagine that torsional stress must induce a major restructuring of chromatin to lead to DNase I sensitivity.

Indeed it is conceivable that the presence of DNase I sensitivity in chromatin is incidental to the existence of torsional stress and does not represent its *raison d'être*. In procaryotes, for example, appropriate levels of torsional stress appear to be important for proper promoter function and regulatory protein interaction (43). Weintraub et al. (49) have proposed a similar role for torsional stress in eucaryotes. It is therefore plausible that the existence of domains of DNase I sensitivity in eucaryotes merely reflects the presence of torsional stress, the principal physiological role for which is related more directly to promoter function itself.

In any event it still remains to be determined whether domains of torsional stress are fully coincident with domains of DNase I sensitivity. The physical size of the β -globin DNase I-sensitive domain has not yet been determined, but it extends over at least 32 kilobase pairs (38). As a first step we are presently engaged in determining the complete extent of DNase I sensitivity surrounding the β -globin genes for comparison to more precise gamma and bleomycin target size analyses of the type described above.

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