Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5505–5509, September 1983
Biochemistry

Transcribed chromatin exhibits an altered nucleosomal spacing

(micrococcal nuclease digestion/nucleosomal repeat lengths/chromatin structure)

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Communicated by Ernest Beutler, June 6, 1983

ABSTRACT **The nucleosomal repeat lengths of bulk chromatin and the chromatin of transcriptionally active and inactive genes were analyzed in two mouse cell lines and adult mouse spleens. The adult β-globin gene exhibits a nucleosomal repeat length approximately 11 base pairs longer than (*i*) an inactive embryonic globin gene, *εy3*; (*ii*) an immunoglobulin heavy chain gene, C_μ; and (*iii*) the bulk chromatin in murine erythroleukemia cell line DS19. The repeat length of the C_μ gene was approximately 14 base pairs longer than that of the adult β-globin or *εy3* genes in the IgM-producing cell line M104E. The chromatin of several inactive genes had repeat lengths less than or equal to bulk chromatin. Individual genes were shown to vary in repeat length among the cell types examined. In addition, genes that exhibited an increased nucleosomal spacing were digested to mononucleosomes more rapidly than bulk chromatin or inactive genes with shorter repeats. Increased repeat length was also correlated with an increased sensitivity to DNase I. Thus, increased nucleosomal spacing may be a property of transcriptionally active genes or genes with the potential for transcription.**

It is evident that transcribed genes are packaged into a chromatin conformation that is different from inactive genes (1–4). Genes that are expressed or have the potential to be expressed exhibit a preferential susceptibility to cleavage by DNase I or micrococcal nuclease (MNase). This enhanced sensitivity is characterized as a reduction of a particular sequence into small nonhybridizable fragments following relatively limited digestion of bulk chromatin. The structural basis of this increased sensitivity is unclear.

One characteristic feature of a given chromatin is the nucleosomal repeat length. Nucleosome cores invariably contain 146 base pairs (bp) of DNA wrapped around a histone octamer. However, the length of DNA that links the cores together for bulk chromatins varies among organisms (5) and tissues (6–10) and may even vary within a particular cell type (11). Differences in the repeat lengths of bulk chromatin as large as 40 bp have been reported between developmental stages of a particular organism (10, 12).

Under specific conditions of digestion, MNase preferentially introduces double-stranded cuts into linker DNA (13) while its exonucleolytic activity can be minimized. Using MNase we have analyzed not only the bulk repeat length in several mouse cell lines but also, with the use of specific DNA sequences as hybridization probes, the repeat lengths of active and inactive genes. Thus, we were able to determine the relationships between the nucleosomal repeat length of a specific gene and its nuclease sensitivity and transcriptional state. Our data suggest that active genes have an increased repeat length compared to inactive genes; this increased spacing may contribute to an increased susceptibility to nuclease attack.

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MATERIALS AND METHODS

Cell Culture. Stock cultures of DS19, a subclone of Friend murine erythroleukemia (MEL) cell line 745 derived from DBA/J2 mice (14) were grown in Eagle's minimal essential media (MEM) supplemented with 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 15% fetal calf serum (Flow Laboratories). Experimental cultures were grown for 72 hr in Falcon culture flasks to densities between 8×10^5 and 2×10^6 cells per ml. Cells were induced by the addition of hexamethylene bisacetamide (HMBA) to a final concentration of 4 mM. Cell viability was determined by trypan blue dye exclusion. The number of cells containing hemoglobin was assayed by benzidine staining. Induced cultures, after 72 hr of induction, routinely contained >95% benzidine-positive cells.

The myeloma cell line M104E (a generous gift of S. McMillan) was grown as an ascites tumor in pristane-primed BALB/c mice. Spleen cells were prepared from DBA/J2 mice from which the DS 19 cell line was originally derived.

Isolation and Digestion of Nuclei for Repeat Length Analysis. Cells were washed three times in C buffer (1 mM Tris·HCl/25 mM KCl/0.9 mM $MgCl_2$/0.9 mM $CaCl_2$/0.14 mM spermidine, pH 7.6) and homogenized in a Dounce homogenizer at 4°C. Nuclei were pelleted, washed twice in C buffer, resuspended at 40 A_{260} units per ml, and digested for 10 min at 4°C with 80 units of MNase per ml (Worthington).

Electrophoretic Analysis of DNA and Hybridization. DNA was purified from the digested nuclei by treatment with 0.2% $NaDodSO_4$ and 150 μg of proteinase K per ml for 3 hr at 37°C, followed by two extractions with phenol, two extractions with chloroform, and precipitation in ethanol. Fifteen micrograms of DNA was loaded onto each lane of 1.7% agarose gels and electrophoretically separated. After ethidium bromide staining, the DNA was transferred to nitrocellulose by the method of Southern (15) and hybridized with radiolabeled DNA probes.

Hybridization probes were labeled with ^{32}P by nick-translation to a specific activity of 10^8 cpm/μg (16). The probe for the adult β-globin sequence was obtained by digesting the plasmid pCR1-βM9 (17), which contains a cDNA copy of mouse β-globin mRNA, with the endonuclease *Hha* I. The fragment bearing the inserted cDNA sequence was then isolated by preparative electrophoresis in agarose gels followed by electroelution. The cDNA probe for *εy3* gene was obtained similarly from a *Hin*cII digest of the pBR322-egz plasmid (18). Preparations of fragments carrying the C_μ immunoglobulin heavy chain gene were obtained from the p104 Eμ_{12} plasmid (a generous gift from J. Gautsch). Hybridizations were performed at 50°C in 30% formamide/50 mM Hepes buffer/0.45 M sodium chloride/0.045 M sodium citrate, pH 7/10 mM EDTA/5× concentrated Denhardt's solution (19) and 400 μg of salmon

Abbreviations: HMBA, hexamethylene bisacetamide; MEL, murine erythroleukemia; MNase, micrococcal nuclease; bp, base pair(s).

sperm DNA per ml for 48 hr. Blots were washed in 0.30 M sodium chloride/0.030 M sodium citrate, pH 7, and 30 mM sodium chloride/3 mM sodium citrate, pH 7, at 37°C. Under our conditions the β-globin cDNA probe does not cross-hybridize with the *εy3* sequence. Autoradiography was performed by using Kodak XRP-1 films and intensifying screens.

Analysis of Repeat Length. To examine the nucleosomal repeat length of specific mouse genes, deproteinized DNA from MNase-digested nuclei was separated by electrophoresis and transferred to nitrocellulose and the immobilized DNA was then hybridized with radioactive probes for specific genes. Migration distances of bands observed in ethidium bromide-stained gels or autoradiographs were measured from densitometric scans. The length of DNA in base pairs for each band was calculated from the mobilities by a modification of the method of Sperling *et al.* (20), as described (21). The data from each experiment were fitted individually by using a least-squares linear regression analysis. The repeat lengths were determined by the slopes obtained from plots of nucleosome number vs. DNA length (base pairs). The mean repeat lengths for bulk chromatin and specific DNA sequences were each calculated from several separate experiments. Comparisons of repeat lengths were made by calculating the critical values of the *t* statistics assuming equal variances of the means.

Nuclease Sensitivity Measurement. Cell nuclei were prepared as described above. MNase digests were performed with 25 units of enzyme (Worthington) per ml at 37°C. DNAse I digests were performed at the same temperature with 20 units of enzyme (Boehringer Mannheim) per ml. These reactions were terminated by ice chilling samples and the addition of EDTA to a final concentration of 20 mM and 0.1% $NaDodSO_4$. Afterwards, DNA from either MNase- or DNAse I-digested chromatin samples was prepared by treatment with 0.2% $NaDodSO_4$ and 100 μg of proteinase K per ml at 37°C for 2 hr. The DNA was further purified by extraction with phenol and chloroform followed by precipitation in ethanol. DNA was resuspended in 10 mM Tris·HCl, pH 7.6/1 mM EDTA and 15 μg of DNA from each sample was bound to nitrocellulose membranes by the method of Robinson *et al.* (22). Hybridization-probe DNA labeled by nick-translation (16) was in excess compared to filter-bound DNA. Hybridizations were performed in 0.9 M sodium chloride/0.09 M sodium citrate, pH 7/Denhardt's solution (19)/0.5% sodium pyrophosphate at 65°C for 48 hr. Filters were extensively washed at 65°C in 0.9 M sodium chloride/0.09 M sodium citrate, pH 7, 0.45 M sodium chloride/0.045 M sodium citrate, pH 7, and finally 0.15 M sodium chloride/0.015 M sodium citrate, pH 7. Radioactivity was determined by scintillation counting.

RESULTS

Repeat Length of Specific Genes in MEL Cell Chromatin. The β-like globin genes in mice are linked and occupy approximately 60 kilobases of DNA (23). The MEL cell line DS19, which possesses potentially active β-globin genes, can be induced to differentiate with HMBA (14, 24). During erythroid differentiation, these cells exhibit a >30-fold increase in adult β-globin messenger RNA production (25). On the other hand, the embryonic globin gene, *εy3*, is not expressed in either the induced or uninduced cell (14).

MEL cell nuclei from uninduced or induced cells were digested with MNase at 4°C to minimize the exonucleolytic activity of the enzyme (13, 21). In preliminary studies, we found that nucleosomes from genes that were more sensitive to MNase digestion (26) were trimmed at a faster rate than nucleosomes from relatively insensitive genes when digestions were performed at 37°C. The extent of digestion was 2–3% based on acid solubility. Under the digestion conditions employed, little or no rearrangement of histone H1 occurs (27, 28). Fig. 1*A* shows the ethidium bromide stain of a 1.7% agarose gel in which MNase-digested DNAs have been separated. After the transfer of this DNA to nitrocellulose, hybridizations with either the labeled β-globin cDNA or *εy3* cDNA probes were performed. The resulting autoradiographs are shown in Fig. 1 *B* and *C*. Fig. 2 shows the densitometer scans of the ethidium bromide-stained gel and the autoradiographs. The relative mobility of each band was used to calculate the length of each fragment (see *Materials and Methods*). The fragment length was plotted as a function of nucleosome number (Fig. 3). The slopes of these lines were taken to be the mean nucleosomal repeat length (20) and are reported in Table 1. The nucleosomal repeat length (mean ± SD) of bulk chromatin in uninduced MEL cells was 175.4 ± 1.9 bp. In induced MEL cells the value was 175.3 ± 2.0 bp. Similar repeat length values were obtained after 60 min of digestion, confirming the minimal amount of exonucleolytic activity of MNase at 4°C (data not shown). As comparisons, the repeat lengths for the embryonic globin gene, *εy3*, which is not transcribed in MEL cells, were determined to be 175.4 ± 1.8 and 175.4 ± 3.3 bp for the uninduced and induced cells, respectively (Table 1). These values were similar to those observed for the mean repeat length of MEL cell bulk chromatin. The same analysis was applied to the Southern blots hybridized with the β-globin probe. However, the repeat length of the β-globin gene chromatin was found to be 185.9 ± 3.3 bp. This difference of approximately 11 bp between the mean repeat lengths of the β-globin and either the *εy3* or bulk chromatin was observed for both induced and uninduced cells (Table 1). Statistical analysis showed that the mean repeat length values for the β-globin and bulk chromatin were significantly different ($P < 0.001$) as were the values for β-globin and *εy3* chromatin ($P < 0.03$).

The densitometric scans in Fig. 2 show that the β-globin gene in both induced and uninduced MEL cells was digested more rapidly to mononucleosomes than was bulk chromatin or the inactive *εy3* gene. Based on the integration of the monomer

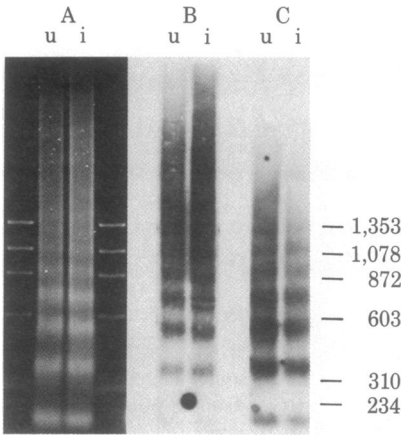

FIG. 1. Electrophoretic analysis of DNA isolated from MNase-digested MEL cell nuclei for the analysis of repeat length. MEL cells were incubated in the presence or absence of 4 mM HMBA for 72 hr of induction. Cell nuclei were isolated and then digested for 10 min at 4°C with 80 units of MNase per ml. Purified DNA from MNase-digested nuclei were subjected to electrophoresis in 1.7% agarose gels. (*A*) Ethidium bromide-stained gel containing uninduced (u) and induced (i) MEL cell DNA. (*B*) Autoradiogram of the DNA in *A* after hybridization with the *εy3* cDNA probe. (*C*) Autoradiogram of the same DNA hybridized with the β-globin cDNA probe. The standards, in base pairs, represent φX174 DNA cut with *Hae* III.

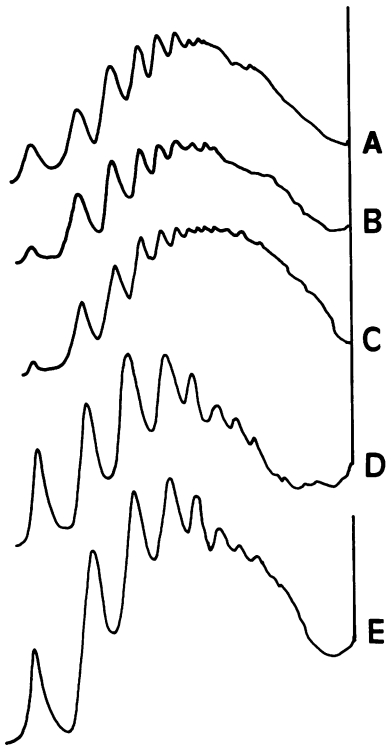

FIG. 2. Densitometric scans of electrophoresis patterns for bulk, β-globin, and εy3 DNAs after MNase digestion for repeat length measurement. After ethidium bromide staining the gel shown in Fig. 1*A* was photographed and the negative scanned in a Kontes Fiber Optic Scanner. The densitometer tracing of DNA isolated from MNase-digested nuclei of the uninduced MEL cells is shown in *A*. The DNA from induced MEL cells has similar scanning patterns (not shown). The autoradiographs of DNA after hybridizations with specific probes from Fig. 1 *B* and *C* were also scanned and their tracings are shown, separately, as follows: (*B*) DNA fragments of uninduced MEL cells hybridized with εy3 probe; (*C*) DNA fragments of induced MEL cells hybridized with εy3 probe; (*D*) DNA fragments of uninduced MEL cells hybridized with β-globin probe; and (*E*) DNA fragments of induced MEL cells hybridized with β-globin probe.

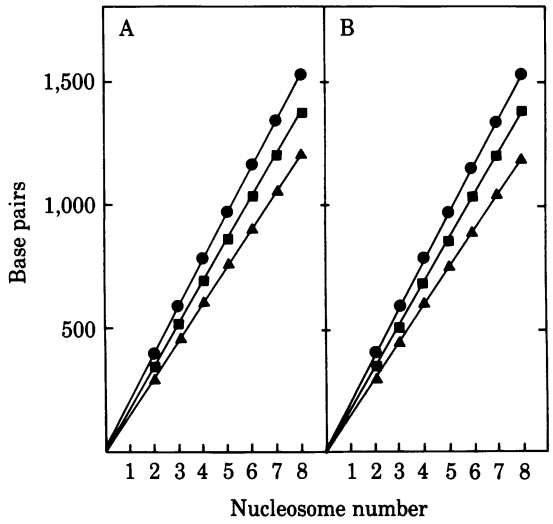

FIG. 3. Analysis of repeat lengths in MEL cells. The nucleosomal repeat lengths were measured (see *Materials and Methods*) and the length of DNA for each fragment was plotted as a function of the number of nucleosomes on each fragment. The data plotted here are some typical values from several experiments, each fitted individually by using linear regression analysis. The slopes of these lines is equal to the mean or average nucleosomal repeat length. Analysis of repeat lengths for uninduced MEL cells is shown in *A* and for induced MEL cells in *B*. ●, β-Globin chromatin; ■, εy3 chromatin; and ▲, C_μ chromatin. The lines for the bulk chromatin in both induced and uninduced MEL cells are similar to those for εy3 gene chromatin (not shown).

peaks, it can be calculated that the rate of monomer production was at least three times faster for the β-globin gene compared to bulk chromatin (compare Fig. 2 *A*, *D*, and *E*). In contrast, the εy3 gene and bulk chromatins had very similar rates of mononucleosome production (compare Fig. 2 *A*, *B*, and *C*). Even though the quantitation of mononucleosomes might suffer from the low blotting efficiency of these DNA fragments, a reliable qualitative estimate of digestion rates can be ascertained based on the relative abundance of monomer, dimer, and trimer vs. higher oligomers of nucleosomes in the gels. Such comparisons of relative abundance among nucleosomes, as shown in Fig. 2, also support the suggestion that β-globin gene chromatin was digested faster than the εy3 gene to form more small nucleosomes. The repeat length values reported here represent an average center-to-center distance for nucleosomes associated with specific gene sequences. It should be noted that the distribution of linker lengths within these particular genes is unclear—that is, it is not yet known whether a constant linker length is maintained in these regions.

Repeat Length of Specific Genes in Nonerythroid Cells. To examine the repeat length of globin gene chromatin in cells that do not exhibit erythropoietic activity, nuclei from unstimulated spleens of adult DBA/J2 mice (29) were digested with MNase under the same conditions and analyzed as described in the preceding experiments. The mean repeat length of bulk spleen chromatin was found to be 190 ± 2.4 bp (Table 1). This value was identical to the 190 ± 2.6 bp obtained for the repeat length of β-globin chromatin in these cells (Table 1). The fact that the bulk nucleosomal repeat lengths vary between MEL cells and spleen cells is not unexpected considering the wide variation of repeat lengths reported for different cell types (5). The difference between the repeat length of the β-globin gene in MEL cells and spleen cells was not statistically significant ($P > 0.05$).

It might be possible that a specific gene, such as β-globin, could maintain a constant repeat length, whereas bulk chromatins exhibit variations of repeat length in a variety of tissues. Alternatively, the similarity of the repeat lengths for the β-glo-

Table 1. Summary of mean nucleosomal repeat lengths in different tissues

Chromatin	Mean repeat lengths*			
	DS19 (HMBA⁻)	DS19 (HMBA⁺)	Spleen	M104E
Bulk	175.4 ± 1.9 (4)	175.3 ± 2.0 (4)	190 ± 2.4 (4)	183.5 ± 2.1 (4)
β-Globin	185.9 ± 3.3 (6)	185.9 ± 3.4 (6)	190 ± 2.6 (2)	182.4 ± 1.1 (2)
εy3	175.4 ± 1.8 (4)	175.4 ± 3.3 (4)	191 ± 1.2 (2)	183.4 ± 2.0 (2)
C_μ	165.0 ± 1.2 (2)	164.8 ± 0.5 (2)	ND	198.2 ± 1.7 (4)

Values are shown as mean ± SD; numbers in parentheses indicate number of experiments. ND, not determined.

*Determined by examining nucleosomes two through eight and reported in base pairs.

bin gene in spleen and MEL cells could be a coincidence. We tested this gene and immunoglobulin heavy chain constant region (C_μ) in an IgM-producing myeloma line, M104E (30). Table 1 shows that the β-globin gene in M104E cells had a repeat length of 182.4 ± 1.1 bp, which was very similar to the bulk repeat length. The β-globin gene in M104E cells had a repeat length significantly different from that in the β-globin gene in both spleen cells (190 bp; $P < 0.005$) and DS19 cells (175 bp; $P < 0.02$). In addition, the C_μ gene had a mean repeat length of 198.2 ± 1.7 bp in M104E cells and 164.8 ± 0.5 bp ($P < 0.005$) in DS19 cells (Table 1). Furthermore, the difference between the C_μ gene in M104E and bulk chromatin was statistically significant ($P < 0.005$). We conclude that neither the β-globin gene nor the C_μ gene has the same repeat length in different tissues. Furthermore, in tissues where these two genes are expressed, they have repeat lengths that are significantly longer than bulk chromatin.

Nuclease Sensitivity of the β-Globin and C_μ Genes. Having established the repeat length of the β-globin and C_μ genes in various mouse cell lines, we next determined the relative sensitivity of these genes to MNase and DNase I. These experiments were performed to examine a possible correlation between repeat length and the accessibility to nuclease attack and to relate these data to the transcriptional status of each gene.

Fig. 4 shows the kinetics of MNase digestion of the β-globin and C_μ genes in either induced MEL cells (Fig. 4*A*) or M104E cells (Fig. 4*B*). After extensive digestions at 37°C (up to 20% acid-soluble DNA), the sensitivity of a particular sequence was characterized by the reduction of the sequence into small, nonhybridizable fragments, as assayed by filter hybridization. In MEL cells the β-globin gene was relatively sensitive to digestion by MNase as compared to the C_μ gene. On the other hand, in M104E cells, the β-globin gene was relatively resistant to digestion compared to the C_μ gene. The unexpressed embryonic globin gene, *εy3*, was resistant to digestion in both cell types, whereas the actively expressed genes (β-globin gene in MEL cells and C_μ gene in M104E cells) showed similar kinetics of digestion. The difference between the rates of digestion for the inactive genes (*εy3* and C_μ genes in MEL cells and *εy3* and β-globin genes in M104E cells) was not significant.

The DNase I sensitivity of these genes was also determined in MEL and M104E cells (Fig. 5) by filter hybridization analysis. In correspondence with results presented in numerous studies, the active β-globin gene was more sensitive to digestion than the C_μ and *εy3* genes in MEL cells and, on the other hand, the C_μ gene was more sensitive compared to the inactive β-globin gene in M104E cells.

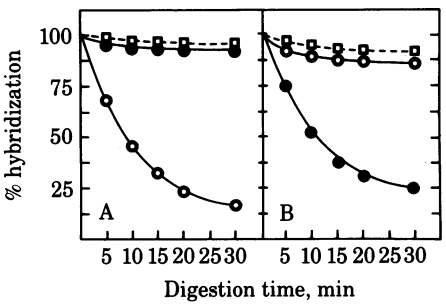

FIG. 4. MNase sensitivity of the β-globin, *εy3*, and C_μ gene sequences. Nuclei were prepared from MEL induced cells (*A*) and M104E cells (*B*) and digested with 25 units of MNase per ml at 37°C for the times indicated. Equal amounts of purified DNA from each digestion time were bound to nitrocellulose filters (22). The series of filters were hybridized with either the β-globin (○), *εy3* (□), or C_μ gene (●) probe. The % hybridization is defined as the percentage of filter bound cpm for each digestion time relative to the number of filter bound cpm for undigested chromatin (100%).

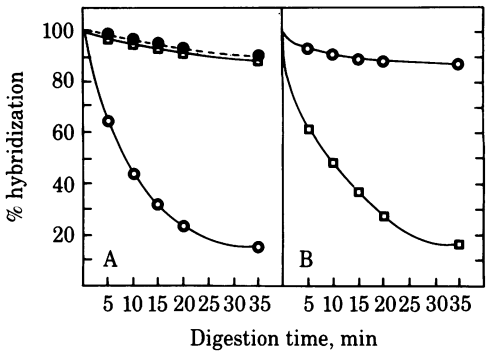

FIG. 5. DNase I sensitivity of the β-globin, *εy3*, and C_μ gene sequences. Induced MEL cell nuclei (*A*) or M104E nuclei (*B*) were prepared and digested with 20 units of DNase I per ml at 37°C. After purification of DNA, filter hybridization was performed and the hybridization probe used was: β-globin (○), *εy3* (●), or C_μ gene (□). The % hybridization was defined as in the legend to Fig. 4.

DISCUSSION

In this report we demonstrate that two genes exhibit a significantly longer repeat length in their active (or potentially active) configurations than in their inactive configurations. The β-globin gene in MEL cells has a mean nucleosomal repeat length approximately 11 bp longer than that of bulk chromatin or of two inactive genes. Similarly, the C_μ gene nucleosome repeat length is approximately 14 bp longer in the IgM-producing, M104E cells.

Individual genes also varied in repeat length in their inactive states among the cell lines examined. Two inactive genes (β-globin and *εy3*) fluctuated in correspondence with the bulk values, whereas the third, the C_μ gene, exhibited a shorter repeat length than the bulk in its inactive state. Thus, a given gene apparently may fluctuate in repeat length according to gene activity of the cell or tissue but, at least for the cases examined here, exceeds the bulk value only when converted to the transcribable configuration.

The increased repeat length of the β-globin gene parallels its nuclease sensitivity in MEL cells. This is also true for the C_μ gene in the M104E cells. Several studies (25, 31, 32) have shown that an active DNase I-sensitive chromatin conformation is not merely a reflection of mRNA synthesis but of an active conformation that is established prior to transcription. Miller *et al.* (33) have reported that the β-globin gene is equally sensitive to DNase I in both induced and uninduced MEL cells and thus is in an active conformation prior to induction. This observation was confirmed in the DS19 cell line used in the present study (data not shown). Because the 186-bp repeat length for β-globin chromatin was found in both induced and uninduced cells, the increased nucleosomal repeat length is not a reflection of RNA transcription but rather an intrinsic structural property of active or potentially active chromatin.

Because the length of the nucleosome cores is invariant (5) the increased nucleosomal repeat length of active genes such as β-globin or C_μ implies an increase in the length of linker DNA between nucleosomes in this region of the genome. One consequence of increased linker length might be an increase in the number of double-stranded cuts introduced into active gene chromatin by MNase. If MNase preferentially digests linker DNA (13), this should result in an increased digestion of active gene chromatin to mononucleosomes relative to bulk chromatin, as is the case of β-globin gene.

Active gene chromatin was digested to nonhybridizable fragments more rapidly than bulk or inactive gene chromatin. Comparisons of such MNase sensitivity among various genes in MEL cells and in M104E suggest a correlation of nuclease sensitivity with the increased repeat length of a particular sequence within the same cell type. However, in addition to the vast difference of the MNase sensitivity between $\varepsilon y3$ and β-globin gene in MEL cells, our recent studies have also demonstrated a distinctive difference of MNase sensitivity between the β-globin gene from induced vs. uninduced MEL cells (unpublished data). Therefore, in addition to the increase in repeat length, other structural features of chromatin must contribute to the further increase in MNase sensitivity of an actively transcribed gene.

In a previous report no difference between the repeat length of bulk chromatin and nucleosomal DNA hybridized with ^{32}P-labeled cDNA was observed (34). It should be noted that this cDNA probe was derived from a diverse population of RNA sequences, some of which are the products of less frequent or very rare transcriptional events, such that the bulk of the signal from the cDNA probe probably reflects genes transcribed at a low level. An interesting example of a particular set of genes having a repeat length different from that of bulk chromatin was reported for the 5S genes in *Xenopus laevis* blood cells (35). The oocyte-type 5S genes, which are not transcribed in blood cells and are present in many copies, had a shorter mean repeat length (176 bp) compared to that in the bulk chromatin (184 bp). These data are similar to the shorter repeat lengths of the inactive gene relative to the bulk chromatin repeat in DS19 cells (Table 1). Together with the findings for $\varepsilon y3$ and C_μ genes in DS19 chromatin and β-globin genes and $\varepsilon y3$ globin in M104E chromatin, they suggest that inactive genes may have a repeat length less than or equal to bulk chromatin.

We thank Michael Riggs and Michael Akong for technical assistance. We also thank Dr. J. Wiktorowicz for assistance with the statistical analysis and Drs. J. Gottesfeld and A. Annunziato for helpful discussions. This work was supported by grants from the National Institutes of Health (AGO2305 to R.L.S. and HL21845 to J.Y.). R.D.S. was supported by a National Institutes of Health postdoctoral fellowship (GM 07904). J.Y. is an Established Investigator from the American Heart Association. This is publication no. 2962 from the Research Institute of Scripps Clinic.

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