between structure and function in the ribosome.

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Chromatin Structure of the 5S Ribonucleic Acid Genes of *Xenopus laevis*†

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ABSTRACT: Staphylococcal nuclease digestion has been used to investigate the structure of bulk chromatin and of that containing the genes for 5S RNA in two tissues from *Xenopus laevis*. In red blood cells, the nucleosome repeat length of the majority of the chromatin is 189 ± 4 bp. In nuclei prepared from liver cells, two populations of nucleosome repeat lengths were detected. The majority of the chromatin has a repeat length of 178 ± 5 bp, but, at late times of digestion with staphylococcal nuclease, a fraction of chromatin with a repeat length of 155 ± 8 bp is apparent. Oocyte-type 5S DNA comprises 0.38% of the *X. laevis* genome, or about 30 000 repeating units per diploid cell, but is not transcribed in somatic tissues. During nuclease digestion, the fate of these sequences has been monitored by hybridization with specific probes. By using the blotting technique of E. M. Southern ((1975) *J. Mol. Biol. 98*, 503–517), the nucleosome repeat length of 5S DNA is 175 ± 5 bp in both blood and liver, suggesting that these specific genes may have a constant chromatin structure, independent of the organization of bulk chromatin in the two tissues. In addition, 5S DNA is less susceptible to staphylococcal nuclease than is bulk DNA in nuclei from both cell types. Quantitative hybridization techniques demonstrate that, with increasing degrees of digestion, high molecular weight nucleosome multimers are enriched up to threefold in 5S DNA sequences. Concomitantly, the 5S DNA content of monomer DNA from these digests is reduced. Calculations indicate that the oocyte 5S DNA sequences, which in the tissues studied are transcriptionally silent, are cut by staphylococcal nuclease at 60–80% of the rate that the bulk DNA is cut.

It is now clear that the nucleosome structures first described in rat liver (Hewish & Burgoyne, 1973; Noll, 1974) and in chicken erythrocytes (Olins & Olins, 1974) can be found as the basic unit of chromatin structure in all eukaryotes (for review, see Kornberg, 1977). Current models envision a repeating array of structures, consisting of 2 molecules each of the histones H2A, H2B, H3, and H4, and with approximately 140 bp of DNA wrapped round the protein core (Sollner-Webb & Felsenfeld, 1975; Axel, 1975; Ramsay-Shaw et al., 1976). This core structure seems to be invariant in size and histone content in eukaryotes (Noll, 1976; Compton et al., 1976; Morris, 1976; Lohr et al., 1977). However, the spacing of the nucleosomes in the chromatin varies considerably in different organisms, from 160 bp in fungi (Noll, 1976; Morris, 1976) to 241 bp for sea urchin sperm (Spadafora et al., 1976), due to different lengths of internucleosome DNA. This DNA, which is associated with the histone H1 (Noll & Kornberg, 1977), is the site of attack by nucleases (Noll, 1974) which cleave to give the characteristic banding pattern of multiples of the monosome DNA size.

It has been suggested that longer internucleosome distances are correlated with transcriptionally inactive chromatin (Morris, 1976; Thomas & Thompson, 1977), perhaps due to differences in the histone primary sequence (Morris, 1976) or to chemical modification of the protein (Thomas & Thompson, 1977; Allfrey, 1971). It is, however, clear that both transcribing and nontranscribing DNA sequences can be found

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in nucleosome structures (Lacey & Axel, 1975; Gottesfeld & Melton, 1978) as well as every specific DNA sequence studied so far, such as the ribosomal genes of *Xenopus* (Reeves, 1976), bovine satellite DNA sequences (Lipchitz & Axel, 1976), and avian ovalbumin genes (Garel & Axel, 1976).

We have investigated the chromatin structure in the nuclei of two tissues of the amphibian *Xenopus laevis* and have compared the nucleosome arrangement of total chromatin with that found associated with a specific DNA sequence, the reiterated genes coding for the small ribosomal 5S RNA. The 5S genes occur in tandemly repeated clusters at the telomeres of most or all of the chromosomes (Pardue et al., 1973). The majority of this DNA codes for the oocyte type of 5S RNA and consists of repeating units of about 700 bp (Brown et al., 1971; Carroll & Brown, 1976a), the primary sequence of which is now fully known (Fedoroff & Brown, 1978; Miller et al., 1978). In the oocyte, 120 bp of the repeat are transcribed into the mature 5S RNA; in somatic cells, the genes are not expressed (Wegnez et al., 1972). Thus, the proteins associated with both inactive and actively transcribing chromatin can be compared on the same DNA sequence. We feel that such structural information is important in our attempts to elucidate the cellular control mechanisms of transcription. It has been suggested recently that the distribution of nucleosomes might be regularly arranged, or "phased" with respect to the DNA sequence (Pondor & Crawford, 1977; Musich et al., 1977; Lohr et al., 1977). The short repeat length of the reiterated 5S genes and recent success in transcribing them in vitro (Parker & Roeder, 1977) make them ideal for investigating the question of the relationship of chromatin structure to function.

Reeves & Jones (1976) have shown that *Xenopus* 5S DNA can be found in the nucleosome structures isolated from *Xenopus* tissue culture cells. We have confirmed and extended their observations, using a hybridization probe that detects both gene and spacer DNA sequences, and have demonstrated that the chromatin structure of 5S DNA has a distinct and similar nucleosome repeat pattern length in the two somatic tissue studies. Analysis of the rate of digestion of *Xenopus* chromatin from the two tissues indicates, however, that the structure of 5S chromatin differs from the majority of the cell chromatin, since staphylococcal nuclease cuts 5S chromatin at a reduced rate compared with that of the majority of the cell chromatin.

Experimental Procedures

Preparation and Digestion of Nuclei. Adult female *Xenopus laevis* were killed and bled into 1 × SET (0.15 M NaCl, 5 mM Tris, pH 7.9, 1 mM EDTA), 5 mM EDTA, 2.5 units/mL heparin for 30–60 min on ice. Blood cells were collected and washed twice in the same buffer by centrifugation at 6000*g* for 5 min. Nuclei were prepared by using a method described by Van Lente et al. (1975). Blood cells were lysed by gentle homogenization on ice, in 10 mM NaCl, 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 0.5% NP40 (Shell Products) (STMN buffer). Ten milliliters of the lysate was layered over 10 mL of 1.0 M sucrose in STMN buffer and spun for 10 min at 1500 rpm in a Sorvall HB 4 rotor at 4 °C. The supernate was decanted, the nuclear pellet resuspended in STMN buffer, and the procedure repeated. Nuclei were suspended in STMN buffer for immediate digestion, or in STMN/50% glycerol, and stored at −20 °C.

Livers were excised, after exsanguination, and stored at −20 °C. Nuclei were prepared as described above but required 3–4 washes to reduce cytoplasmic contamination. Concentration of DNA was estimated from the A_{260} of an aliquot of the nuclei in 0.02 N NaOH, by assuming an A_{260} = 1 corresponds to 35 μg/mL. Digestion with staphylococcal nuclease (Worthington) was carried out at 37 °C in a shaking water bath. Aliquots were taken at appropriate times, and the reaction was stopped by the addition of EDTA to 5 mM. For DNA size analysis, $NaDodSO_4$ was added to 0.5%, samples were deproteinized by extraction with phenol, and DNA was precipitated by the addition of NaCl to 0.5 M and 2 volumes of ethanol. Samples were prepared for gel electrophoresis and run in a 3.0% polyacrylamide–0.5% agarose vertical gel, as described by Carroll & Brown (1976a). Following electrophoresis, the gels were stained and photographed as described (Carroll & Brown, 1976a).

To prepare chromatin size fractions, nuclease digested nuclei were rapidly mixed with an equal volume of glycerol, followed by EDTA to a final concentration of 2 mM, and Tris (pH 7.4) to a concentration of 10 mM. The sample was further diluted, if necessary, and stored at −20 °C at about 200 μg/mL of DNA. A 3–4.5-mL portion of this sample was loaded onto a Bio-Gel A-5m column (Bio-Rad), 2.5 × 90 cm, in 10 mM Tris (pH 7.5), 0.7 mM EDTA (Ramsay-Shaw et al., 1976). Two-milliliter samples were collected at a flow rate of about 12 mL/h, the A_{260} of each was read, and the appropriate samples were pooled. Following deproteinization with phenol, ethanol precipitation, and desalting through Sephadex G-25, aliquots were denatured in 0.02 N NaOH and scanned, by using a Beckman Model 25 spectrophotometer to estimate DNA concentration. Samples for hybridization were lyophilized with the addition of 10–20 μg of *E. coli* tRNA carrier.

Total DNA samples were prepared from blood cells or liver cell nuclei, by using an $NaDodSO_4$/Pronase method as described by Brown et al. (1971). High molecular weight DNA samples for hybridization were sonicated as described below before final purification.

Preparation of Hybridization Probes. Plasmids were maintained and prepared by standard procedures, according to the NIH guidelines governing recombinant DNA research (July 7, 1976), and by using P2 + EK1 containment. The recombinant plasmid used, pHU1054, was constructed as described by D. Carroll and C. C. T. Simonsen (manuscript in preparation). It consists of 5 tandem repeats of the 5S gene and spacer DNA in a segment that was isolated from its original pSC101 vector (Carroll & Brown, 1976b) and recloned in pBR313 (Rodriguez et al., 1976). Strains carrying pBR313 and pMB8 were gifts of Dr. Herb Boyer. Plasmid DNAs were prepared as described by Clewell & Helinski (1969). λ DNA (cI857S7) was prepared from a heat-induced lysogen.

For in vivo labeling, thymidine-requiring *E. coli* strain Ymelsup$^+$3 (a kind gift of Dr. Costa Georgopoulos) was transformed to ampicillin resistance with pHU1054 (Cohen et al., 1972). These transformants were grown to an A_{650} of 0.5, in M9 minimal medium supplemented with thymidine to 5 μg/mL and casamino acids to 0.35%. Chloramphenicol was added to 200 μg/mL; the cells were collected by centrifugation and then resuspended in 0.5 volume of medium with chloramphenicol, but without thymidine. [^{3}H]Thymidine (New England Nuclear), 40–60 Ci/mmol, was added to 0.2 μg/mL and the cells were shaken at 37 °C. Similar amounts of [^{3}H]thymidine were added at three 1-h intervals, and the culture was then shaken overnight. Plasmid DNA was prepared by using the cleared lysate procedure (Clewell & Helinski, 1969). From an original 500-mL culture of cells, 80 μg of plasmid pHU1054, with a specific activity of 1 × 10^6 cpm, was recovered.

The restriction enzyme *Eco*RI was prepared according to Greene et al. (1974). *Bam*HI was purchased from New England Biolabs, Beverly, MA. Co-digests of *Eco*RI and *Bam*HI were performed at 37 °C, usually for 2 h, in 50 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 6 mM Tris (pH 7.4). Preparative 1% agarose gels were run as previously described (Carroll & Brown, 1976a,b) with up to 100 μg of a *Bam*HI and *Eco*RI digest of pHU1054. After staining briefly in 1 μg/mL ethidium bromide, the appropriate region of the gel was excised and the DNA recovered as described below. Strand separation of DNA fragments was carried out on 2% agarose gels in phosphate buffer at 4 °C as described by Hayward & Smith (1972).

DNA was extracted from agarose gels by dissolving the agarose with 2–3 volumes of 5 M sodium perchlorate at 60 °C for 5 min. DNA was adsorbed to hydroxylapatite (Bio-Rad), and the agarose was removed by washing with 0.03 M phosphate buffer (pH 6.8). DNA was eluted with 0.4 M phosphate buffer, 2.5 μg/mL *E. coli* tRNA (Sigma) and precipitated by addition of ethanol. Phosphate was removed by desalting through a Sephadex G-50 column in 10 mM Hepes (pH 7.0), 50 mM NaCl, containing a pad of Dowex chelating resin (Sigma), and the DNA was reprecipitated with ethanol. DNA samples for hybridization were sonicated in 0.1 N NaOH for 3 × 15 s, by using a Branson sonifier. This reproducibly reduced the single-stranded length to 500–700 bases. The solution was then neutralized by the addition of 1.0 M Hepes (pH 7.0) and HCl, desalted through Sephadex G-25 in water, and lyophilized. For hybridization in solution, samples were incubated at 39 °C in a sealed, silicone-treated capillary in a volume of 4 μL of hybridization buffer (0.5 M NaCl, 25 mM Hepes, 0.5 mM EDTA, pH 6.8), 50% formamide (Mallinckrodt), 25 μg/mL *E. coli* tRNA (Harrison et al., 1974). The hybridization reaction was stopped by flushing out the capillary with 350 μL of nuclease buffer (70 mM sodium acetate, 2.8 mM $ZnSO_4$, 140 mM NaCl (pH 4.5), 10 μg/mL denatured calf thymus DNA (Worthington)). One hundred microliters of each sample was precipitated with 5% trichloroacetic acid to measure the total radioactivity present; 200 μL of each sample was digested with 7 units of nuclease S_1 (Sigma) for 2 h at 37 °C, acid precipitated, filtered through Millipore filters (45-μm pore size), and counted in toluene-based scintillant.

For blot hybridization, transfer of the DNA to Millipore filters and subsequent treatment and hybridization were as described by Southern (1975). A ^{32}P-labeled cRNA probe was prepared as described by Brownlee et al. (1974) by using the 5S DNA containing fragment cut from pHU1054 as template and [α-^{32}P]UTP (20–200 Ci/mmol, New England Nuclear) as labeled triphosphate; 1 × 10^6 cpm were incubated in a chamber containing 2 mL of 2 mM EDTA, 1% $NaDodSO_4$, 6 × SSC (1 × SSC is 0.015 M sodium citrate, 0.15 M NaCl, pH 7.9) at 68 °C for 16 h, with the nitrocellulose filter. Filters were washed extensively in 6 × SSC at 68 °C, briefly in 2 × SCC, and treated for 30 min at room temperature with 50 μg/mL pancreatic RNase A (Worthington). Following further extensive washing at room temperature in 2 × SCC, the filters were dried and exposed for autoradiography, using Kodak X-ray film SB-5.

5S DNA was prepared from adult *Xenopus* ovaries by the method of Brown & Brown (1976). A 5-μg portion was labeled with ^{125}I, by a method similar to that described by Prensky (1976), to a specific activity of 3 × 10^7 cpm/μg.

Results

The Nucleosome Size of Xenopus Blood and Liver Chromatin. When *Xenopus* liver or blood nuclei were digested with staphylococcal nuclease, an increasing percentage of the DNA was rendered acid soluble. As shown in Figure 1, the DNA in liver chromatin was less susceptible to nuclease attack than in blood chromatin. Liver nuclei were digested more slowly and, reproducibly, about 10% more of the DNA is protected at the plateau level. In the absence of added nuclease, no significant increase in DNA acid solubility above the zero time levels could be detected (not shown).

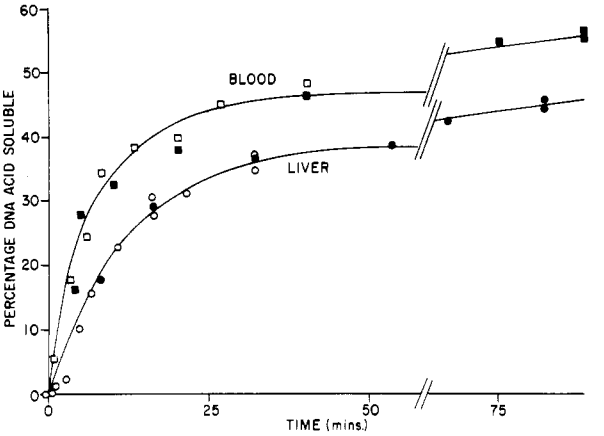


FIGURE 1: Digestion of blood and liver nuclei with staphylococcal nuclease. Nuclei from blood cells (□) or from liver cells (○), at a DNA concentration of 500 μg/mL, were digested with 300 units/mL (open symbols) or 900 units/mL (closed symbols) of staphylococcal nuclease. At appropriate times, samples were removed and the percentage of the DNA rendered acid soluble was calculated by a diphenylamine estimation (Burton, 1956) of duplicate aliquots taken before and after acid precipitation. The curves are corrected to 100% acid precipitability at zero time by subtraction of the zero-time background (25% for the blood samples and 16.5% for the liver samples). The reason for the high zero-time levels of acid solubility is unknown, but was reproducible (±3% for the blood nuclei) in four experiments. Data from the low concentration of nuclease are plotted on the same scale after dividing the time of digestion by 3 (Clark & Felsenfeld, 1974).

The size of the DNA fragments generated during the early stages of nuclease digestion (up to about 15–20% acid solubility) was examined next. Following digestion for the appropriate times with low concentrations of nuclease, DNA fragments were deproteinized, ethanol precipitated, and analyzed by gel electrophoresis as shown in Figure 2. Bands representing multiples of a nucleosome monomer can be seen in digested samples from both blood and liver nuclei. DNA in nuclei incubated under the same conditions, in the absence of added nuclease for up to 1 h, did not show a significant reduction in size, nor a nucleosome repeat pattern (not shown). The sizes of the DNA fragments from the chromatin digests were estimated, by using restriction fragments of λ DNA and pMB8 DNA run in the same gel, as shown in Figure 2. The values for the DNA repeat lengths in the blood and liver nuclei were calculated from Figure 3 by taking the slope of the graph of band size (in base pairs) against band number (Noll & Kornberg, 1977; Thomas & Thompson, 1977). From several such analyses, the average size of the DNA repeat lengths observed at early (5 min, 1–2% acid solubility) and late (60 min, 15–20% acid solubility) times of digestion of blood nuclei are both 189 ± 4 bp (the mean of six independent determinations). The early digestion pattern (1–2% acid solubility) from liver nuclei gives a repeat size of 178 ± 5 bp, but at late times (10–15% acid solubility) the repeat size was reduced to 155 ± 8 bp (the mean of eight independent determinations).

Although the reason for the generation of this different pattern is not clear, several experiments were performed to

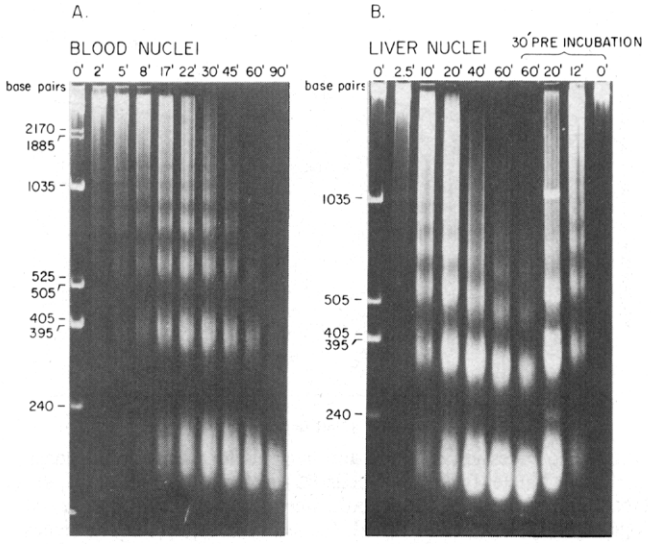


FIGURE 2: Size analysis of DNA fragments from chromatin digests. Three percent polyacrylamide, 0.5% agarose composite gels were run at 40 V (25 mA) for 12 h and stained with ethidium bromide as described. (A) Stain pattern of DNA fragments from digested blood nuclei. Nuclei at a concentration of 200 μg of DNA/mL were digested with 100 U/mL of staphylococcal nuclease for the time indicated. The zero-time sample was mixed with marker DNA of a *Hin*dIII digest of λ DNA and a *Hae*III digest of pMB8 DNA. The sizes of the marker DNA restriction fragments have been previously determined by electron microscope measurements (Carroll & Brown, 1976a) or by comparison of their electrophoretic mobility with known length standards (D. Carroll, unpublished). (B) Stain pattern of DNA fragments from digested liver nuclei. Nuclei at a concentration of 200 μg of DNA/mL were digested with 50 U/mL of nuclease for the indicated times (lanes 1–6). Samples in lanes 7–10 were preincubated at 37 °C for 30 min, then 50 U/mL nuclease was added, and samples were taken at the times indicated. Samples in lanes 1 and 8 were mixed with marker DNA of a *Hae*III digest of pMB8.

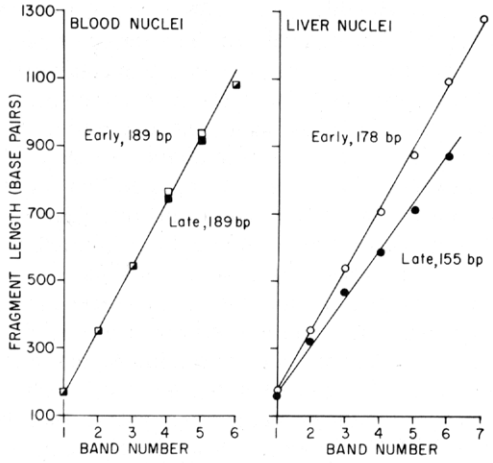


FIGURE 3: Determination of DNA repeat lengths in chromatin of *Xenopus* blood and liver. The sizes of DNA bands from blood (□) and liver (○) nuclei were estimated from gels such as shown in Figure 2, by using restriction fragments from pMB8/*Hae*III and λ/*Hin*dIII as calibration standards. Open symbols represent data from early digests and closed symbols data from late digests of the two tissues. Distances of migration were measured to the midpoints of the gel bands. The DNA repeat length is given by the slope of the least-squares regression line fitted to the data.

rule out artifactual causes. Electrophoretic artifacts were ruled out by running length standards in the same slots as the chromatin derived DNAs (not shown). Another possibility examined was that the ionic conditions of the digestion buffer were allowing sliding and rearrangement of the chromatin proteins. Liver nuclei were thus preincubated at 37 °C in STMN buffer for 30 min before the addition of nuclease, and the size of the DNA fragments generated was analyzed as

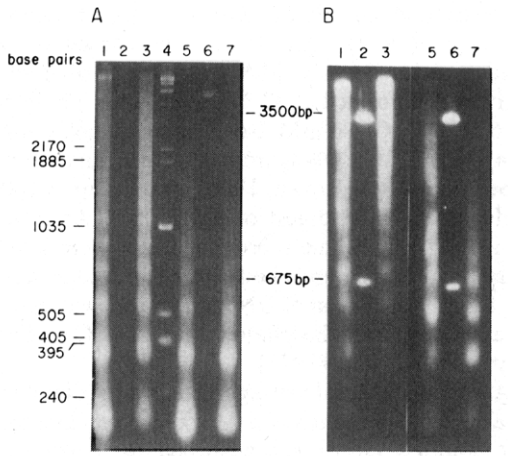
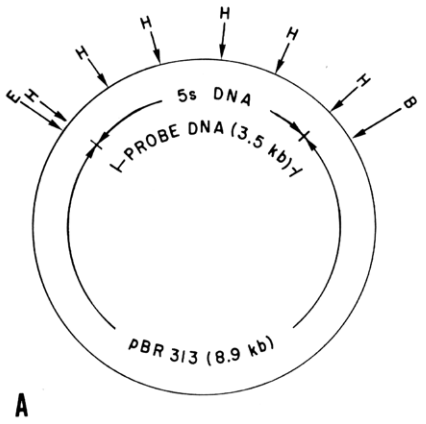


FIGURE 4: Size comparison of the DNA fragments in nuclease digested total chromatin and 5S chromatin. (A) Stain pattern of digested fragments on 2% agarose gel; (lane 1) 6 μg of an early blood nuclei digest; (lanes 2 and 6) 0.025 μg of a *Hin*dIII digest of pHU1031 that contains a single 5S DNA repeat of 675 bp (D. Carroll, unpublished) plus 0.012 μg of unlabeled 3500 bp 5S DNA from pHU1054; (lane 3) 6 μg of an early liver nuclei digest; (lane 4) a size marker of λ DNA/*Hin*dIII with pMB8/*Hae*III; (lane 5) 7 μg of a late blood nuclei digest; (lane 7) 7 μg of a late liver nuclei digest. (B) Autoradiogram of 5S cRNA hybridization. DNA fragments from lanes 1–3 and 5–7 of the above gel were transferred to nitrocellulose and hybridized with cRNA as described. The hybridization of cRNA to the 3500 bp and 675 bp 5S DNA (lanes 2 and 6) was used to align the autoradiogram with the stain pattern. The molecular weights of the chromatin derived 5S DNA fragments were estimated by using the λ/*Hin*dIII and pMB8/*Hae*III restriction fragments (lane 4, above).

before. Figure 2B demonstrates that preincubation had no effect on the rate of generation of the smaller repeat length fragments. Similarly, the observed pattern of DNA fragments from blood nuclei was not altered by preincubation (not shown).

The Nucleosome Size of Xenopus 5S Chromatin. The effect of nuclease digestion on chromatin containing 5S genes (5S chromatin) was next determined. DNA was extracted from blood and liver nuclei that had been digested for different times with staphylococcal nuclease. This DNA was run on a 2% agarose gel, and the gel was stained and photographed, as shown in Figure 4A, in order to visualize the nucleosome repeat pattern. The DNA was then transferred to nitrocellulose filters as described by Southern (1975), and the filters were hybridized with ^{32}P-labeled cRNA, specific for 5S DNA sequences. The resulting autoradiogram is shown in Figure 4B. Because small fragments of DNA do not bind quantitatively to nitrocellulose filters (Southern, 1975; Botchan et al., 1976), the monosome band is underrepresented in the cRNA hybridization compared with the stain pattern. However, it is clear that the 5S genes are available to nuclease attack, resulting in a similar nucleosome repeat pattern in the autoradiogram as seen in the stained gel. Regression analyses (not shown) give values of 175 ± 5 bp (mean of six determinations) for the 5S chromatin monomer from both blood and liver digests—similar in length to the majority of the liver chromatin repeat pattern, but considerably smaller than that calculated in Figure 3 for blood chromatin. The 5S nucleosome repeat length is not significantly smaller in extensive digests of liver nuclei.

Preparation of a Single-Stranded Hybridization Probe. More information on the structure of 5S chromatin was obtained from a comparison of the rate of digestion of 5S and non-5S chromatin by staphylococcal nuclease. For quantitative analysis, a single-stranded hybridization probe suitable for solution hybridization studies was prepared by in vivo labeling



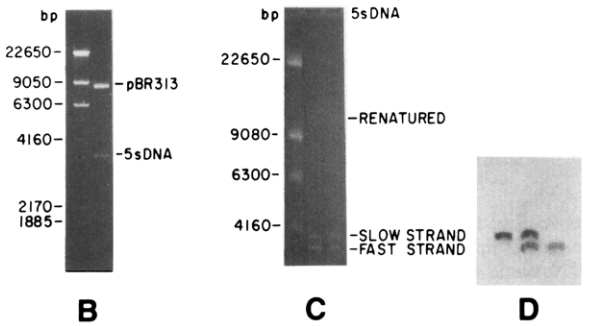


FIGURE 5: (A) Restriction enzyme map of the plasmid pHU1054. Sites for the restriction enzymes *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B) are indicated. (B) Preparation of 5S DNA containing fragment. One percent preparative agarose gel; (lane 1) λ DNA marker digested with *Hin*dIII; (lane 2) 0.5 μg of an *Eco*RI + *Bam*HI digest of pHU1054. (C) Preparation of 5S DNA single strands. Two percent agarose gel in phosphate buffer; (lane 1) denatured λ DNA marker digested with *Hin*dIII; (lanes 2 and 3) 0.75 μg of 5S DNA plasmid fragment. DNA at a concentration of 6 μg/mL in 10 mM NaCl, 2.5 mM Tris, pH 7.9, 1.0 mM EDTA was denatured by the addition of NaOH to 0.1 N, at 0 °C for 15 min. A 0.1 volume of 0.01% bromophenol blue in electrophoresis buffer was added, aliquots were loaded on the gel (about 0.75 μg/slot), and the samples were run rapidly into the gel (200 V, 140 mA, 10 min). Electrophoresis was then at 75 V (55 mA) for 19 h with recirculation of the buffer. Staining and photography was as described. (D) Blot hybridization to separated strands. A gel like that in C was blotted (Southern, 1973) and separate lanes were hybridized with ^{125}I-labeled 5S RNA (left), ^{32}P-labeled 5S cRNA (right), and a mixture of the two (center).

of a recombinant DNA plasmid pHU1054, containing a 5 repeat fragment of 5S DNA as shown in Figure 5A. As seen in Figure 5B, digestion of the plasmid with a mixture of *Eco*RI and *Bam*HI excised a 3500-bp fragment which contains the 5S DNA and about 350 bp of plasmid sequences (D. Carroll and C. C. T. Simonsen, manuscript in preparation). This DNA was recovered from a preparative agarose gel and denatured, and the strands were separated on a second agarose gel, as shown in Figure 5C, as described by Hayward & Smith (1972). Recovery of these separated strands gave a hybridization probe that is complementary to the entire coding and spacer sequence of the 5S DNA. Hybridization studies, using either strand as probe, gave identical results (not shown). In some preparations, the slow migrating strand resolved into two bands. The reason for this is unclear, but may be due to slight heterogeneity in the plasmid stock or result from two secondary structure forms that can be adopted by this DNA fragment. In blot hybridization experiments, ^{125}I-labeled 5S RNA hybridized exclusively with the slow migrating strand, which therefore represents the DNA strand transcribed in vivo

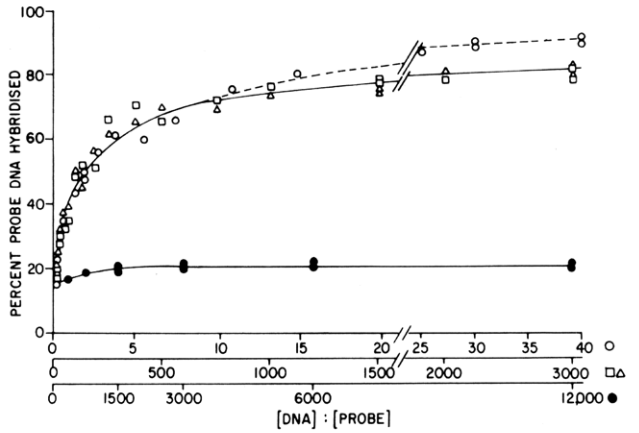


FIGURE 6: Hybridization of 5S DNA probe to plasmid and total *Xenopus* DNAs. One nanogram of DNA probe (about 1000 cpm) was incubated with increasing amounts of DNA sample in 4-μL aliquots for 24 h. This represents a probe D_0t of 0.072 mol s L^{-1} which is ~20 times the $D_0t_{1/2}$ of the probe alone. The titration curve for the plasmid pHU1054 DNA was first determined. To estimate the proportion of 5S DNA sequences in each DNA sample, the abscissa was adjusted to superimpose the data on the plasmid curve. (○) pHU1054 DNA (% 5S DNA 3500 × 100/12 500 = 28%). (□) Total blood cell DNA (0.375% 5S DNA). (△) Total liver cell nuclear DNA (0.375% 5S DNA). (●) λ DNA. The number of 5S genes per haploid genome can be estimated as follows: no. of 5S genes = (no. of base pairs of DNA per haploid genome × % of DNA complementary to 5S DNA probe)/average size of 5S DNA repeat. The haploid amount of DNA is 3 pg (Dawid, 1965) or 2.74 × 10^9 bp. The number of 5S genes is thus [(2.74 × 10^9 bp/haploid genome) × 0.0038]/700 bp/5S gene = 14 900 5S genes per haploid genome.

(Figure 5D). In the same experiment, [^{32}P]cRNA made in vitro from a native 5S DNA template by *E. coli* RNA polymerase hybridized only with the faster, noncoding strand, consistent with previous findings (Reeder, 1973).

We have used a titration curve analysis (Harrison et al., 1974; Young et al., 1974) to estimate the 5S DNA sequence content of *Xenopus* DNA. In this approach, equal amounts of probe DNA were incubated with decreasing amounts of sample DNA. Use of this type of analysis has been rigorously justified by Young et al. (1974), provided the hybridization reaction has gone to completion. A titration curve of probe DNA incubated with plasmid pHU1054 at 39 °C for 24 h is shown in Figure 6. Incubation of the hybridization samples for 24 or 48 h gave identical titration curves with plasmid DNA (data not shown), demonstrating that by using this probe concentration (0.25 μg/mL), the hybridization reaction was complete after 24 h. The $D_0t_{1/2}$ (the product of initial DNA concentration and time required to achieve 50% of complete reassociation) for double-stranded 5S probe DNA was 0.0075 mol s L^{-1} (S. Humphries, unpublished). By comparison with the reassociation of double-stranded globin cDNA (Young et al., 1974), the base sequence complexity of the 5S probe DNA is about 1000 bp. This is similar to the expected value for the 700-bp repeating unit of 5S DNA, plus about 350 bp of the plasmid DNA.

The hybridization studies shown in Figure 6 demonstrate the specificity of the probe. In the absence of added DNA, 15–20% of the probe sequences became S_1 resistant during incubation at 39 °C for 24 h. Significant hybridization above this background level did not occur to phage λ DNA, even at very high DNA:probe ratios nor to *E. coli* DNA (data not shown). However, the probe hybridized to greater than 90% to plasmid DNA at appropriate concentrations and to total *Xenopus* DNA from blood or liver to greater than 80%. This slightly lower plateau for *Xenopus* DNA is expected since it should not contain the approximately 10% of the probe which

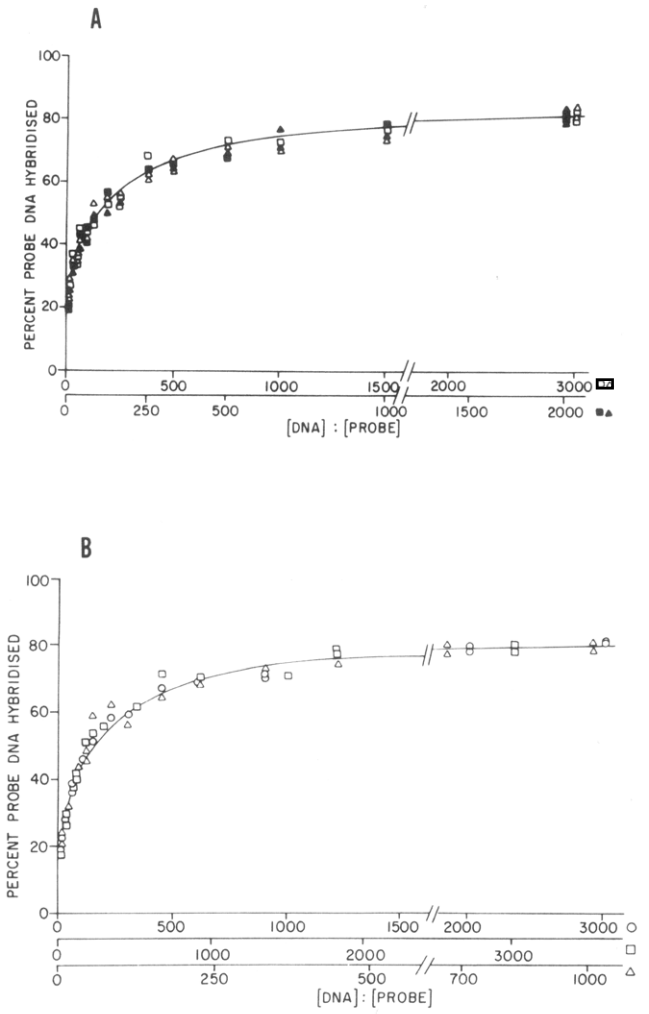


FIGURE 7: The 5S DNA titrations of DNA from chromatin digests. Hybridizations with the DNA probe were performed as described for Figure 6. The line shown in both A and B is the titration curve generated by the data from Figure 6 for total blood and liver DNA, plotted on the first abscissa (0–3000). The values in parentheses are the percentages of the DNA sample estimated from the titration curves to be proportional to 5S DNA sequences. (A) Hybridization of DNA fragments from unfractionated chromatin digests. (□) Early digest of total blood chromatin, 6% of DNA acid soluble (0.38% 5S DNA). (△) Early digest of total liver chromatin, 4% of DNA acid soluble (0.38%). (■) Late digest of total blood chromatin, 16% of DNA acid soluble (0.46–0.57%). (▲) Late digest of total liver chromatin, 10% of DNA acid soluble (0.46–0.57%). (B) Hybridization of DNA fragments from fractionated chromatin digests. DNA from a late liver digest (10% acid solubility) was purified and hybridized with the DNA probe as described. (△) Pool I (high molecular weight DNA; 1.05% 5S DNA). (○) Pool II (intermediate molecular weight DNA; 0.38%). (△) Pool III (low molecular weight, monomer DNA; 0.25%).

comes from the plasmid sequences. This result also demonstrates that, if a DNA sample is deficient in as little as 10% of the sequences of the 5S DNA repeat, this would be detectable as a decrease in the titration curve plateau level. Since the titration curves obtained for *Xenopus* DNA are otherwise almost superimposable upon the probe:plasmid DNA curve, the proportion of 5S DNA sequences in the *Xenopus* samples can be estimated from the ratio of the sample abscissa to the plasmid abscissa (Humphries et al., 1976). The absolute number of 5S genes can be calculated, by assuming that the plasmid contains 28% 5S DNA (3500 × 100/12500 bp) and that the average size of a 5S DNA repeat is 700 bp (Carroll & Brown, 1976a). From Figure 6 it can be calculated that *Xenopus* DNA contains about 0.38% 5S DNA or 15000 genes per haploid genome.

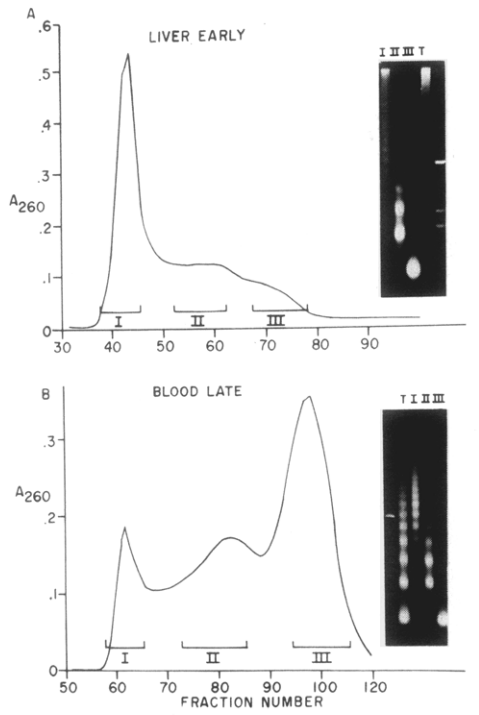


FIGURE 8: Size fractionation of blood and liver chromatin fragments on Bio-Gel A-5m. (A) Six hundred micrograms of an early liver digest (4% DNA acid soluble) and (B) 900 μg of a late blood digest (16% DNA acid soluble) were loaded onto a Bio-Gel A-5m column and fractions collected as described. Fractions were combined as indicated, deproteinized, and ethanol precipitated. Five to seven micrograms of the total digest (T), each of the fractions I, II, and III and a size marker of pMB8/*Hae*III were run on a 2% agarose gel as described.

Sensitivity of 5S Chromatin to Staphylococcal Nuclease. By using this approach, it was possible to estimate the percentage of 5S DNA in the nuclease-digested samples. Titrations of the DNA from early and late digests of blood and liver nuclei were performed and the results are shown in Figure 7A and Table I. If the 5S DNA is in a chromatin structure identical with that of bulk DNA, it should be digested at the same rate, and the proportion of the DNA that is complementary to the probe should remain constant at 0.38%. However, it can be seen from Figure 7A that this is not so. At early times of digestion (4–6% DNA acid soluble), the titration curve of probe hybridization was indistinguishable from that of total blood or liver DNA plotted on the same abscissa. But at a time when 10–16% of the DNA has been rendered acid soluble, the hybridization data were only superimposable on the total DNA titration curve when the scale of the abscissa was reduced by a factor of about 1.2–1.5-fold. Thus, the proportion of hybridizable 5S DNA sequences in these late chromatin digests is higher than 0.38% by a factor of 1.2–1.5-fold. This suggests that, in blood and liver chromatin, the nuclease digests 5S DNA at a slower rate than the majority of the cell DNA, to fragments that are too small to hybridize to the probe.

A more sensitive confirmation of this reduced rate of 5S chromatin digestion was carried out as shown in Figure 8, by using chromatin size fractions prepared as described by Ramsay-Shaw et al. (1976). Digested chromatin was separated on a Bio-Gel A-5m column into excluded, high molecular weight chromatin fragments, containing DNA of greater than 1000 bp, an intermediate size fraction corresponding to a DNA size of 400–1000 bp and monosome length chromatin fragments. In our hands, this method can easily be adapted to handle milligram quantities of chromatin digests and can resolve small fractions of the total genome DNA, with their

Table I: Analysis of 5S DNA Content of Nuclease Digested Chromatin DNA Fractions

DNA pool	% of total DNA in each pool[b]	fract of DNA comp to probe/ 0.38%[c]	col 2 × col 3[d]	calcd fract of DNA comp to probe/ 0.38%[e]	obsd fract of DNA comp to probe/ 0.38%[f]
very early digest of blood nuclei (1–2% of DNA acid soluble)					
I[a]	56.5	1.0	56.5		
II	30.4	1.0	30.4	1.00	not done
III	13.1	1.0	13.1		
early digest of liver nuclei (4% of DNA acid soluble)					
I	45.8	1.25	57.25		
II	28.5	1.0	28.5	1.07	1.0
III	26.7	0.8	21.4		
late digest of blood nuclei (16% of DNA acid soluble)					
I	16.5	3.0	49.5		
II	34.4	1.7	58.5	1.42	1.2–1.5
III	49.1	0.7	34.4		
late digest of liver nuclei (10% of DNA acid soluble)					
I	21.7	2.75	59.7		
II	32.2	1.0	32.2	1.21	1.2–1.5
III	44.1	0.67	29.5		

[a] The Roman numerals I, II, and III refer to the different size fractions of chromatin DNA, prepared as described in Figure 8. [b] The percentage of DNA in each pool was estimated from the A_{260} profiles shown in Figure 8 by integration, by using an electronic graphics calculator (Model 1224, Numonics Corp.), or by summation of the amounts of DNA in the appropriate fractions. The methods gave similar values to within 5%. [c] Estimated from titration curves, such as shown in Figure 7B. Because of the inherent limitations of the hybridization analysis, the values shown are probably only accurate to within 0.09% 5S DNA. [d] Column 2 × column 3 gives the distribution of 5S DNA sequences in the three DNA fractions. These values are used to estimate the relative rate of 5S DNA cleavage (see below). [e] These values are a summation of the data in column 4 and are thus estimates, from the fractionated samples, of the proportion of 5S DNA sequences in the total blood and liver digests. [f] Data from the hybridizations shown in Figure 7A. The observed proportion of 5S DNA in total early and late digests is thus in close agreement with the calculated values from the fractionated digests of these samples. By assuming that the distribution of sites for staphylococcal nuclease cleavage is random on the available DNA, the relative rates of nuclease cutting for the 5S DNA and the total DNA were determined as follows: if p = extent of digestion (number of cuts) and x = nucleosome multiple band number, then the fraction of the DNA in an $x$mer (W_x) is given by the formula $W_x = xp^2(1-p)^{x-1}$. For the simple case of $x = 1$ (monomer or pool III DNA), this reduces to $W_1 = p^2$, and, therefore, $p(5S)/p(\text{total}) = [(W_1(5S)/W_1(\text{total})]^{1/2}$. For the late blood and liver monomer DNA, $p(5S)/p(\text{total}) \simeq 0.8$, indicating that 5S DNA is cut at 80% the rate of the total DNA. For pool I DNA, where $x > 4$, the equation becomes $W = \Sigma_{x=5}^{\infty} xp^2(1-p)^{x-1}$. A computer program was used to convert the data in Table I to values of $p(5S)/p(\text{total})$, which were 0.61 and 0.56. We consider these values to be the more reliable estimates of the rate of 5S DNA cutting. Because of the inherent inaccuracies in the hybridization analysis, the higher values of 5S DNA sequence content observed for the high molecular weight DNA fractions are more significant than the lower values of monomer DNA.

associated proteins. DNA fragments from the three molecular weight fractions were purified and analyzed for 5S DNA sequence content. The high molecular weight DNA was sonicated to reduce its single strand size to 500–700 bases prior to hybridization with probe. Titrations of the DNA from a fractionated late digest of liver chromatin are shown in Figure 7B, and the results from several such analyses are summarized in Table I. It can be seen that, as digestion proceeds, the high molecular weight DNA from both liver and blood nuclei became progressively enriched in 5S DNA sequences, by a factor of up to threefold. Concomitant with this, the observed proportion of 5S DNA in the monomers decreased to 70–80% of the value for total *Xenopus* DNA. The decrease in the observed proportion of 5S DNA in the monomers from late digests is not due to a decrease in the hybridizability of small fragments of DNA. Sonicated blood DNA of a mean size of less than 250 bases gave an identical titration curve to blood DNA of mean length of 500–700 bases (data not shown). Furthermore, Table I shows that monomer DNA prepared from an early digest of liver DNA contained the expected 0.38% of 5S DNA sequences, and the single-strand molecular weights of early and late digested monomer DNA were identical (not shown).

These data indicate that, during staphylococcal nuclease digestion of blood or liver nuclei, the reiterated 5S genes are cleaved into monomer DNA fragments at a slower rate than the majority of the cell DNA. We have attempted to make a quantitative estimate of the reduced rate of digestion of the 5S chromatin from the data in Table I. Our calculations, shown in Table I, indicate that the 5S chromatin is cut at 60–80% of the rate at which the nuclease cuts the majority of the cell chromatin.

Discussion

As has been previously reported (Reeves & Jones, 1976), the nucleosome structures of *Xenopus* chromatin are similar to those found in other eukaryotes. Our analyses for *Xenopus* blood and liver nuclei give nucleosome repeat lengths that are slightly shorter than those reported for chicken erythrocytes (212 ± 5 bp), chicken liver (200 ± 5 bp) (Morris, 1976), rat liver (198 ± 6, Noll & Kornberg, 1977), and mouse liver (195 ± 5 bp, Gottsfeld & Melton, 1978). However, the relative difference we observe between the repeat pattern length for blood and liver chromatin is similar to that seen in these same tissues in the chicken.

It has been noted that an increased length of nucleosome spacing correlates with a decreased transcriptional activity of the chromatin. For example, within a single tissue, the rabbit brain, the nucleosome repeat size varies from 160 bp in transcriptionally active cerebral cortex cell to 200 bp in transcriptionally inactive glial cells (Thomas & Thompson, 1977). It has been suggested that the different nucleosome spacings observed may reflect the varying concentration of basic amino acids found in some of the H1 proteins associated with the internucleosome DNA of different species (Noll, 1976; Morris, 1976). In agreement with this, lysine and arginine rich H5 histone found in chicken erythrocytes is associated with a longer nucleosome repeat length than is observed in chicken liver, where the less basic histone H1 is found.

For *Xenopus*, the primary sequence of the histones is not known, although Biroc & Reeder (1976) have shown that the H1 proteins are more complex than those of calf thymus and that *Xenopus* erythrocytes contain several additional bands in the H1 region of the gel, which may correspond to histone H5. It is puzzling that *Xenopus* blood nuclei should be more sensitive to staphylococcal nuclease digestion than liver nuclei as shown in Figure 1. This may reflect structural differences in the association of the proteins with the internucleosome DNA or may simply result from a higher permeability of blood cell nuclear membrane to the enzyme. Alternatively, the faster rate of digestion may result from greater DNA availability in the longer nucleosome repeats we observe in blood cells. This may also explain why more of the DNA in blood nuclei can be digested to acid soluble products, since a greater proportion of the total DNA is outside of the protected "core" particle than in the liver chromatin.

As the digestion proceeds in the liver nuclei, but not the blood nuclei, the predominant DNA bands have a lower repeat value of about 155 bp as compared with 178 bp at early digestion times. A similar phenomenon has been observed in Physarum (Johnson et al., 1976) where the average repeat length is reduced upon extensive digestion from 190 bp to 172 bp. As Johnson et al. suggest, these observations are consistent with the presence of two types of chromatin with different nucleosome spacing. The majority of the chromatin exhibits a long repeat pattern with large internucleosome bridge regions that are highly susceptible to nuclease attack and are cleaved early in digestion. A small percentage of the DNA is in a less susceptible, short repeat length form, where cleavage of the smaller internucleosome region only occurs after extensive digestion. These smaller repeat fragments then predominate in the high molecular weight region of the gel, to give the repeat pattern observed. It is possible that the small repeat length chromatin fraction may represent chromatin that is being actively transcribed in the liver, with the transcriptionally silent chromatin being packaged in a long repeat form, although a recent report (Gottesfeld & Melton, 1978) indicates transcriptionally active and inactive DNA sequences in the same tissue may be packaged in similar repeat length nucleosomes. Another possibility is that the short repeat length chromatin is found exclusively in a subpopulation of liver cells, such as occurs in the different cell types of the rabbit brain (Thomas & Thompson, 1977).

The size of the nucleosome repeat of 5S chromatin was next examined. Since these genes are highly reiterated in all cells (Brown et al., 1971), the sequences can be readily detected in blot hybridizations. In both blood and liver chromatin, the size of the 5S nucleosome repeat is 175 ± 5 bp. The 5S nucleosome repeat length is thus distinctly shorter than the repeat length observed for the blood cell chromatin, although it is of a similar size to the major fraction of the liver chromatin repeat length. In an extensive digestion of liver chromatin, the 5S nucleosome repeat is not significantly reduced in length. Thus, even though, in the two somatic tissues studied, the chromatin proteins are complexed with the majority of the DNA to give significantly different nucleosome repeat lengths, the chromatin proteins associated with the 5S genes give a similar structure in the two tissues. These observations are consistent with the idea that the 5S genes may be present in both tissues in a similar chromatin structure that is distinct in some way from the majority of the cell chromatin.

Since small fragments of DNA, such as monosome DNA sequences, are not efficiently retained on nitrocellulose filters during hybridizations (Southern, 1975; Botchan et al., 1976), it was necessary to develop a solution hybridization assay to accurately quantitate the rate of digestion of 5S chromatin. From the titration curves in Figure 6, the proportion of 5S DNA in total *Xenopus* DNA can be calculated. Both *Xenopus* blood and liver DNAs contain about 0.38% 5S DNA, or 15 000 genes per haploid genome. This value is about one-half the previously reported values (Brown et al., 1971). A likely explanation for this is that these authors used in vivo labeled 5S RNA, which will hybridize to both the gene sequences and the pseudogene sequences which also occur once every 5S gene repeat (Jacq et al., 1977).

Using this method to measure the 5S DNA content of staphylococcal nuclease digests of liver and blood nuclei, we found evidence that 5S chromatin is digested more slowly than is bulk chromatin. With increasing degrees of digestion, the total chromatin DNA becomes enriched in 5S sequences. This is presumably because some non-5S DNA has been digested to small or acid-soluble fragments that are lost during purification of the DNA. When the chromatin digests are fractionated, it is observed that the high molecular weight (>5 nucleosome) DNA is up to threefold enriched in 5S sequences, while the nucleosome monomer DNA is depleted. In all cases, the plateau and shape of the titration curves are identical with those for total *Xenopus* DNA, indicating that gene and spacer sequences are equally available to the nuclease. Analysis of the hybridization results suggests that 5S DNA in chromatin is digested at about 60% the rate of bulk DNA, in both blood and liver nuclei.

These results clearly demonstrate that the availability of the 5S DNA to staphylococcal nuclease is reduced, when compared with its action on the rest of the chromatin, both with respect to its ability to cleave the internucleosome DNA and subsequently to digest the internucleosome DNA to acid-soluble products. This strengthens the idea that the 5S chromatin is structually different in some way from the bulk of the chromatin in both blood and liver nuclei. Several recent reports have suggested that DNA sequences that are being actively transcribed are more sensitive to nuclease digestion than nontranscribed sequences. Thus, during nuclease digestion, monomer sized DNA fragments become enriched, while high molecular weight DNA fragments become reduced in the proportion of DNA sequences that are being transcribed in the cell (Johnson et al., 1978; Bloom & Anderson, 1978). In both the *Xenopus* tissues we have analyzed, the 5S genes are transcriptionally silent. In red blood cells, very little transcription is occurring (Cameron & Prescott, 1963) and, in liver cells, the bulk of the DNA is probably also transcriptionally silent (for example, see Chikaraishi et al., 1978). It thus appears that 5S chromatin is less sensitive to nuclease digestion even when compared with bulk chromatin that is also transcriptionally silent. At the present time, the chromatin components, presumably chromatin proteins or modified chromatin proteins that are responsible for the reduced sensitivity to nuclease digestion of the 5S DNA in somatic chromatin, have not been identified. The *Xenopus* 5S genes are only actively transcribed in the developing oocyte, and it will be of interest to study the nucleosome size and nuclease sensitivity of the 5S DNA in this tissue.

One explanation for our data that we have not experimentally excluded is the possibility that the 5S DNA sequences are inherently less susceptible to staphylococcal nuclease digestion than the majority of the DNA sequences. This might be because of the base composition or chemical modification, such as methylation (Fedoroff et al., 1978). However, we feel that this possibility is unlikely. It has been reported that

staphylococcal nuclease shows a slight preference for digesting A-T rich sequences in native DNA (Wingert & Von Hippel, 1968). However, the titration curves from samples of DNA that are either enriched or depleted in 5S DNA sequences are superimposable with data from hybridization with undigested total DNA samples. This indicates that both the regions of high G-C and high A-T content that comprise the 5S DNA repeat (Brown et al., 1971) are equally represented in digested samples and are, thus, equally available to nuclease attack.

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