Articles

Efficient Solubilization and Partial Purification of Sea Urchin Histone Genes as Chromatin†

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ABSTRACT: Soluble chromatin fragments are rapidly and efficiently produced when nuclei are digested with restriction endonucleases in buffers containing very low concentrations of magnesium. Under these conditions, the sequence specificity of the restriction endonucleases is maintained, ılting in release of specific genes as fragments with discrete molecular weights that can be fractionated by size on glycerol gradients. Gradient fractions can be chosen to be significantly enriched in specific genes and their associated proteins. For instance, we can achieve a 16-fold enrichment of the chromatin containing the early histone genes of sea urchin. The enrichments produced by these methods are useful as a first step in techniques to purify specific genes as chromatin. Glycerol gradient analyses can also be used to test whether putative gene-specific proteins are actually bound to the same sequences in vivo.

Since the discovery of restriction endonucleases and the development of recombinant DNA techniques, several approaches have been devised to indirectly probe the structure and composition of specific genes as chromatin. Specifically, recombinant DNA probes have been used to describe nuclease sensitivities of specific genes and to identify hypersensitive sites to nucleases in and near specific genes. These nuclease sensitivities have been related to the functional state of the genes (Weisbrod, 1982; Elgin, 1981; Mathis et al., 1980). Restriction endonucleases have been used directly on cellular chromatin and viral minichromosomes to assay the sensitivity of restriction sites to digestion. The relative accessibilities of these sites have been interpreted in terms of nucleosome-free regions (Varshavsky et al., 1978, 1979; Tack et al., 1981; McGhee et al., 1981), nucleosome phasing on the DNA (Lipchitz & Axel, 1976; Brown et al., 1977; Das et al., 1979; Polisky & McCarthey, 1975; Liggins et al., 1979), the presence of regulatory proteins (Bryan et al., 1983), and nucleosome migration under different conditions (Beard, 1978; Severin et al., 1982).

Restriction endonucleases have also been useful in the enrichment of specific genes as chromatin. Lica & Hamkalo (1983) enriched chromatin containing satellite sequences 6.8-fold by removing nonsatellite chromatin from metaphase chromosomes. Zhang & Horz (1982) and Lipchitz & Axel (1976) produced 70% pure fractions of satellite chromatin by similar techniques. Reynolds et al. (1983) used a combination of restriction endonucleases to achieve a 15–30-fold enrichment for *Xenopus* 5S RNA chromatin.

Chromatin fragments generated with restriction endonucleases have two characteristic features. First, specific genes are released as a characteristic set of fragments of specific lengths. Second, the termini of the specific gene fragments are adjacent to a characteristic set of specific sequences. We have taken advantage of the first characteristic to enrich particular genes up to 16-fold on preparative glycerol gradients. We have taken advantage of the second characteristic to hybridize DNA probe molecules to the restricted chromatin fragments and subsequently perform affinity chromatography to separate specific genes from the bulk of the chromatin. Together, these methods can be used to enrich over 1800-fold for specific gene-containing chromatin.

In this paper, we describe the production of chromatin fragments by restriction endonuclease digestion under conditions optimized for both efficient digestion and maximum solubility of intact high molecular weight chromatin, and also the enrichment of specific genes as chromatin by glycerol gradient size fractionation. These enriched fractions can serve as substrates for the highly sequence-specific nucleoprotein hybridization technique presented elsewhere (Workman & Langmore, 1985).

Our goal has been the isolation of the chromatin containing the tandemly repeated early histone genes of *Strongylocentrotus purpuratus*. The methods presented here are generally applicable toward the study and enrichment of other genes, since they do not utilize any special feature of the chromatin other than the specific distribution of restriction sites.

Materials and Methods

Reagents. Recombinant plasmid pSp102 was kindly supplied by L. Kedes. This plasmid contains 4 kilobase pairs (kbp)[1] of the early histone gene repeat (Cohn et al., 1976). Concentrations of enzymes used in this work are in units (as measured by the supplier) per microgram of DNA, standardized to the assay conditions of BRL. Reagents were screened to test for protease activity by incubating an excess

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; kbp, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TBE, Tris–borate/EDTA; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTT, dithiothreitol; SSC, 150 mM NaCl and 15 mM sodium citrate (pH 7.0).

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of the reagent with chicken erythrocyte nuclei for several hours at 37 °C followed by SDS–PAGE. Enzymes or other reagents which altered the location or intensity of protein bands were not used. PMSF and iodoacetate were used to inhibit endogenous proteases and nucleases during nuclear preparations. Iodoacetate is an irreversible thiol alkylating agent which has been shown to inhibit thiol proteases from chromatin (Tsurugi & Oyanagi, 1980). The inclusion of iodoacetate is absolutely necessary during isolation of nuclei from tissues that have high levels of thiol proteases, such as amphibian erythrocytes (our unpublished results). In the absence of iodoacetate, sea urchin embryo nuclei aggregated during isolation, resulting in reduced yields of soluble chromatin.

Preparation of Sea Urchin Embryo Chromatin. Adult *S. purpuratus* were injected with 1 mL of 0.5 M KCl, and gametes were collected in synthetic seawater (containing, per liter, 24.72 g of NaCl, 0.67 g of KCl, 1.36 g of $CaCl_2 \cdot 2H_2O$, 4.66 g of $MgCl_2 \cdot 6H_2O$, 6.29 g of $MgSO_4 \cdot 7H_2O$, and 180 mg of $NaHCO_3$). Ten milliliters of eggs was fertilized in 400 mL of synthetic seawater containing 1 mM 3-amino-1,2,4-triazole (Showman & Foerder, 1979). After two washes with 400 mL of synthetic seawater, embryos were incubated at 15 °C as a 1% suspension with gentle stirring in synthetic seawater with 100 mg/L streptomycin sulfate. For labeling of cells, 1 mCi of [^{3}H]thymidine or [^{3}H]leucine per liter was added 5 min after the start of the incubation.

At 18 h, embryos were chilled to 4 °C and washed once in 15 mM NaCl, 60 mM KCl, 15 mM Hepes (pH 7.3), 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA, 1 mM iodoacetate, 0.1 mM PMSF, and 0.3 M sucrose by pelleting at 50*g*. They were then resuspended in 24 mL of the same buffer, homogenized with 50 strokes of a Teflon pestle homogenizer, and mixed with 2.1 volumes of the same buffer containing 2.3 M sucrose. Homogenates were layered over 1 mL of this buffer containing 2.3 M sucrose and centrifuged for 20 min at 40 000 rpm in a SW40 rotor at 4 °C.

Nuclear bands were pooled and washed twice in 60 mM KCl, 15 mM NaCl, 15 mM Pipes (pH 6.5), 3 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM iodoacetate, and 0.1 mM PMSF by resuspension and pelleting 10 min at 1000*g*. Nuclei were then washed once without Nonidet P-40 or iodoacetate. Nuclei were used for restriction digestions or stored in buffer containing 30% glycerol at −20 °C.

For restriction endonuclease digestion, nuclei were very gently pelleted and resuspended to A_{260} = 2 in 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. Restriction digestions were done with 5 units/μg of DNA for 3 h at 37 °C, unless otherwise noted. After digestion, nuclear debris was removed by centrifugation for 30 min at 1000*g*, leaving 20–50% of the chromatin in the supernatant.

Preparation of Chicken Erythrocyte Chromatin. Chicken erythrocyte nuclei were prepared according to Langmore & Paulson (1983). Nuclei were first lysed by resuspending to A_{260} = 2.2 in 0.2 mM EDTA (pH 7.0) in order to rupture the tough cytoskeleton of the plasma membrane. One-tenth volume of 660 mM potassium acetate, 330 mM Tris–acetate (pH 8.0), 5 mM magnesium acetate, and 5 mM DTT was added dropwise while gently mixing. Digestions were done at 5 units/μg for 3 h at 37 °C unless otherwise stated. Nuclear debris was then removed by centrifugation for 30 min at 1000*g*. Usually 50% of the chromatin remained in the supernatant state.

Preparation of SV40 Minichromosomes. Minichromosomes, of SV40 strain 776, were prepared by a modification of the methods of Varshavsky et al. (1976). Nuclei from three 9-cm plates were resuspended in 300 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM iodoacetate, and 0.1 mM PMSF. After 1 h at 4 °C, the nuclei were pelleted for 10 min at 10000*g*. The supernatant was loaded onto a 13.5-mL, 15–30% (v/v) glycerol gradient containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and was centrifuged 200 min at 40 000 rpm in a Beckman SW40 rotor. Gradient fractions containing the major rapidly sedimenting band of SV40 were pooled and dialyzed into 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 1 mM 2-mercaptoethanol. Restriction digestions of SV40 minichromosomes were done after dilution into an equal volume of the same buffer with 2× the desired final magnesium concentration.

Chromatin Concentration. Restriction chromatin solutions were placed in 10-mm Spectrapore 2 dialysis bags and the ends sealed with Spectrum Medical Industries closures. The bags were then dialyzed rapidly against 60% sucrose, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. After 1.5 h of dialysis, the clips were moved to constrict the remaining volume, followed by dialysis into 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. A 4-fold concentration with over 90% yield was achieved by this method, without loss of protein or change in the sedimentation velocity of the chromatin.

Glycerol Gradient Fractionation and Detection of Specific Genes. A 0.2–0.3-mL sample of soluble restricted chromatin was loaded on 13.5-mL 15–30% (v/v) glycerol gradients with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Gradients were centrifuged 200 min at 40 000 rpm and 4 °C in a Beckman SW40 rotor. Fractions of 225 μL were collected from the top of the gradients by using a gradient fractionator and an absorbance monitor at 4 °C.

Aliquots (200 μL) of each gradient fraction were denatured by adding an equal volume of 1 M NaOH and 4 M NaCl. After 1 h, the samples were renatured by adding $^2/_3$ volume of 1.5 M HCl and 0.5 M Tris-HCl (pH 8.0) and immediately transferred to presoaked (6× SSC) nitrocellulose on a Schleicher & Schuell Minifold. After sample application, each well was rinsed with 0.5 mL of 6× SSC followed by a brief rinse of the entire filter in 2× SSC. Filters were then baked in a vacuum oven 4 h at 80 °C.

Filters were prehybridized for 3 h at 68 °C in 3× SSC, 10× Denhardt's (Denhardt, 1966), 0.1% SDS, and 50 μg/mL denatured salmon sperm DNA. Hybridization with nick-translated pSp102 (prepared to $>10^8$ dpm/μg) was carried out for 8–12 h in 3× SSC, 1× Denhardt's, 0.1% SDS, and 50 μg/mL denatured salmon sperm DNA at 68 °C. After hybridization, the filters were washed twice for 1 h at 68 °C in 2× SSC, 0.5% SDS, 50 μg/mL denatured DNA, and 20 mM NaH_2PO_4, once in the same solution with 1× SSC, and finally for 1 h in 0.1× SSC, 0.1% SDS, and 50 μg/mL denatured DNA. Filters were autoradiographed at −70 °C. ^{32}P bound for each dot was determined by scintillation counting. Standard amounts of sea urchin sperm DNA and plasmids containing histone sequences were included on each filter.

Electrophoresis and Southern Blots. Samples were diluted in 0.2 volume of 50% glycerol, 1% SDS, and 10% 2-mercaptoethanol and treated for 0.5 h with 1 μg/mL Proteinase K or extracted with phenol and chloroform, precipitated with ethanol, and resuspended in TBE with 5% glycerol. DNA samples were electrophoresed on 0.7% agarose–TBE gels according to Maniatis et al. (1982).

Southern blots were carried out according to Schleif & Wensink (1981) including 1-h exposure of ethidium bromide stained gels to 254-nm ultraviolet light to uniformly nick the

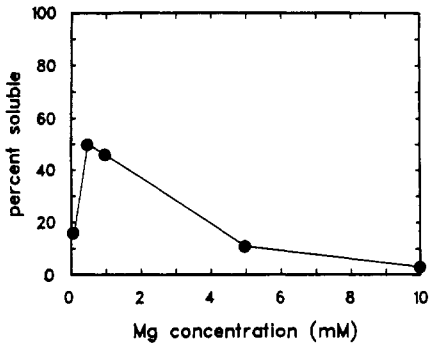

FIGURE 1: Effect of magnesium concentration on the release of chromatin fibers from chicken erythrocyte nuclei by *Eco*RI digestion. Chicken erythrocyte nuclei were digested with *Eco*RI as described under Materials and Methods. The magnesium concentrations in the digestion buffer were 0.1, 0.5, 1, 5, and 10 mM. The percentage solubilized was determined from the A_{260} in the supernatant.

DNA. Nitrocellulose filters were processed as before. Autoradiograms were scanned with a Joyce-Loebl Mark III microdensitometer. The areas under the densitometric scans were calculated to better than 5% accuracy by using a program written for an Apple Macintosh computer utilizing the computer's mouse to trace the scans.

Calculation of Cutting Efficiencies. Assuming the accessible restriction sites are randomly distributed, the probability, P_n, of creating an oligomeric fragment of n tandemly linked repeating units is expressed $P_n = p^2(1-p)^{n-1}$ where p is the probability that any specific site is cleaved. In other words, the probability of creating an n-mer is the probability of having two terminal cuts, p^2, times the probability of having $n-1$ uncut sites, $(1-p)^{n-1}$. In principle, an empirical value for P_n could be determined for each resolved band on the Southern blot and could be used to calculate p. P_n is equal to N_n/N_{tot}, where N_n is the number of fragments of length n and N_{tot} is the total number of fragments of all lengths. Unfortunately, calculation of N_{tot} depends on accurate knowledge of the molecular weight distribution of the high molecular weight fragments, which is impractical due to the poor resolution of the gels. It is practical, however, to determine the ratio of the numbers of fragments in resolved bands, N_{n+1}/N_n, which is equal to P_{n+1}/P_n and therefore equal to $1-p$. Our values of p were calculated from measurements of N_2/N_1 and are in agreement with those calculated from N_3/N_2, validating the use of the statistical model.

RESULTS

Effect of Magnesium on Restriction of Chromatin. Several investigators have developed schemes to produce soluble chromatin fibers with restriction endonucleases (Tack et al., 1981; Varshavsky et al., 1978; Zhang & Horz, 1982; Miller et al., 1978; Pfeiffer et al., 1975; Igo-Kemenes et al., 1977). These methods utilized conditions originally optimized for digesting naked DNA and involved extraction of the chromatin fibers at low ionic strength, often aided by homogenization or agitation. We have optimized conditions for directly releasing chromatin fibers during digestion. To achieve high yields, the nuclei must be made permeable to high molecular weight chromatin (as described under Materials and Methods), and the chromatin must be soluble.

Magnesium is required for the activity of type II restriction endonucleases (Wells et al., 1981). On the other hand, divalent cations cause precipitation of chromatin in nuclei (Langmore & Paulson, 1983) and in solution (Ausio et al., 1984). Therefore, magnesium is expected to limit the solubility of restricted chromatin and perhaps limit the access that the

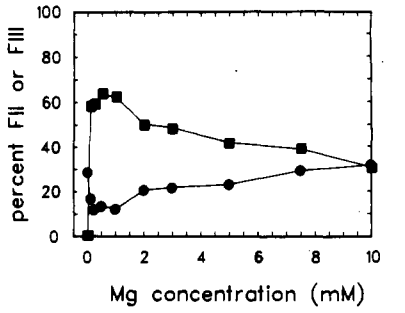

FIGURE 2: Effect of magnesium concentration on the digestion of SV40 minichromosomes with *Kpn*I. SV40 minichromosomes were digested with 5 units/μg of DNA of *Kpn*I for 3 h at 37 °C in buffer containing the magnesium concentrations shown. After digestion, the samples were added to $^1/_5$th volume of 50% glycerol, 1% SDS, and 10% 2-mercaptoethanol and then electrophoresed in TBE on a 0.7% agarose gel. The photographic negative of the ethidium bromide stained gel was scanned, and the peaks were integrated as described under Materials and Methods. Shown is the percent of the SV40 as form II (●) and form III (■) after digestion.

enzymes have to the chromatin. In order to optimize the release of chromatin from nuclei, we studied the digestion of nuclei at different concentrations of magnesium. Figure 1 shows the measured release of chromatin from chicken erythrocyte nuclei by *Eco*RI. Not surprisingly, there was a strong dependence of solubilization upon magnesium concentration, with an optimum found at about 0.5 mM Mg, a substantially lower concentration than the 5 mM Mg usually recommended for digestion of naked DNA (Wells et al., 1981). A control incubation (at 0.5 mM Mg) without added enzyme yielded less than 1% soluble chromatin, demonstrating that the release had not been due to an endogenous nuclease.

Is the observed magnesium dependence due exclusively to the increased solubility of chromatin fragments in low magnesium, or is it also due to an increase in the frequency that restriction sequences are cleaved by the nuclease? To answer this question, we studied the digestion of SV40 minichromosomes. SV40 minichromosomes remain soluble and unaggregated at high concentrations of magnesium, as determined by measurement of the radioactivity remaining in solution and of the sedimentation velocity (data not shown). SV40 cleavage by *Eco*RI, *Kpn*I, and *Bam*HI was studied by agarose gel electrophoresis. All enzymes gave similar results.

Figure 2 shows the magnesium concentration dependence of cleavage by *Kpn*I. At zero time, the minichromosomes were 72% covalently closed, 28% nicked, and less than 1% linear molecules. In high magnesium, the 3-h digestion produced about 30% linear SV40, in quantitative agreement with the results of Shelton et al. (1980). However, after digestion at 0.5 mM magnesium, about 63% of the SV40 minichromosomes were linear, showing that more efficient cleavage had taken place. From the amounts of form I and form II before and after digestion, it became obvious that the supercoiled and relaxed minichromosomes were cut with similar efficiencies in low Mg, whereas only supercoiled SV40 minichromosomes were cut at high Mg. Although there is no definite explanation for this result, the greatly enhanced ability of restriction enzymes to cut relaxed chromatin at low salt might explain the efficient release of cellular chromatin under the same conditions.

Control digestions confirmed that the restriction results for SV40 and cellular chromatin were not kinetically limited by insufficient amounts or denaturation of the enzymes, or by changes in chromatin structure. Figure 3 shows that the conversion of minichromosomes to linear molecules goes to completion in the first minute of digestion at 0.5 mM Mg.

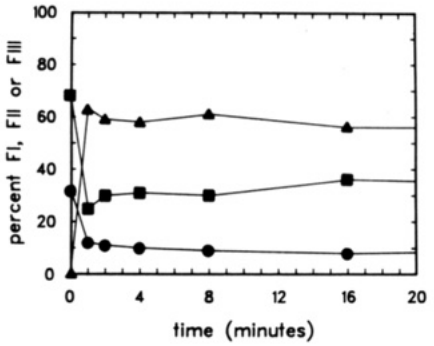

FIGURE 3: Time dependence of *Kpn*I digestion of SV40. Minichromosomes were digested at 37 °C with 20 units/μg of DNA of *Kpn*I in digestion buffer containing 0.5 mM $MgCl_2$. Aliquots were quenched at the indicated times by the addition of EDTA to 25 mM. The aliquots were electrophoresed and analyzed as described in the legend of Figure 2. Shown is the percentage of form I (■), form II (●), and form III (▲) during digestion. There was no further change in the relative amounts of the forms out to 3 h of digestion.

Thus, progressive exposure of sites by a nucleosome-sliding mechanism was not detected. Increasing the amounts of restriction enzyme did not increase cleavage of either cellular or SV40 chromatin (data not shown).

We conclude that low concentrations of magnesium promote efficient release of chromatin from nuclei, probably by not only increasing the solubility of the cleaved fragments but also increasing the efficiency of cleavage, especially of nonsupercoiled chromatin. The very large amounts of enzyme and exhaustive extraction procedures that other investigators [e.g., see Varshavsky et al. (1978), Lica & Hamkalo (1983), Zhang & Horz (1982), Miller et al. (1978), Pfeiffer et al. (1975), and Igo-Kemenes et al. (1977)] found necessary to release chromatin from nuclei or to cleave specific sites in soluble chromatin can be attributed to the high Mg concentrations (3–10 mM) they employed.

Site-Specific Cleavage of Sea Urchin Histone Genes. To use gradient centrifugation and nucleoprotein hybridization to enrich for specific genes, the chromatin must be solubilized as fragments of discrete size or sizes, with sequence-specific termini. We have used restriction enzymes at low magnesium concentrations in order to achieve this goal with the early histone genes of sea urchin. In *S. purpuratus*, all five histone genes are organized into a 6.5-kbp tandemly repeating unit that comprises about 0.25% of the genome (Cohn et al., 1976). Partial restriction of these genes with enzymes specific for only one site per repeat should release integral multiples of the basic unit. The fidelity of the actual cutting pattern to this expectation was used as a criterion of the sequence specificity of the restriction of nuclei. The relative amounts of monomeric, dimeric, and trimeric molecules were used as a measure of the cutting frequencies at the specific sites.

Sea urchin nuclei were digested with *Hin*dIII, *Sma*I, and *Sal*I (single cutters) and with *Bgl*II (a null cutter). After digestion, the nuclei were pelleted and extracted overnight with 0.2 mM EDTA in order to solubilize additional chromatin. Material not soluble at both ionic conditions was then pelleted. DNA from the restriction supernatant, the low-EDTA supernatant, and the final nuclear pellet was electrophoresed, transferred, and hybridized as described under Materials and Methods. Figure 4 shows the resultant autoradiogram.

Control lanes containing sea urchin sperm DNA were digested to completion as the 6.5-kbp monomer by each single-cutting enzyme. No discrete bands were produced by *Bgl*II. The restriction endonuclease digestions of nuclei produced only discrete fragments indicating that there was little

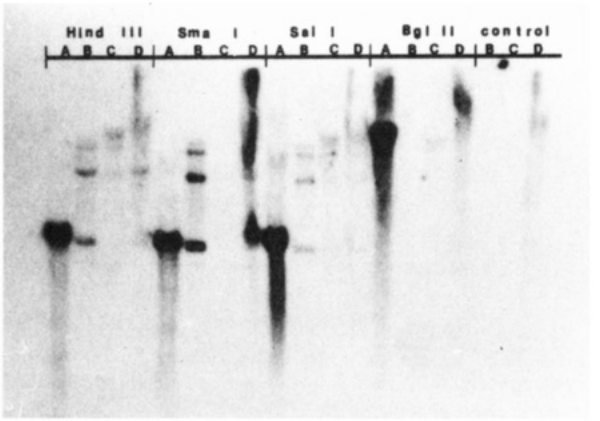

FIGURE 4: Digestion of the early histone gene repeat in sea urchin embryo nuclei. Nuclei were incubated with *Hin*dIII (5 units/μg of DNA), *Sma*I (5 units/μg of DNA), *Sal*I (2 units/μg of DNA), and *Bgl*II (2 units/μg of DNA) and without enzyme. For the *Sma*I digestion, KCl was substituted for NaCl in the digestion buffer. All samples were phenol and chloroform extracted, electrophoresed, and blotted as described under Materials and Methods. For each enzyme, lane A was a sea urchin sperm DNA, lane B was chromatin released directly into digestion buffer, lane C was chromatin not released in digestion buffer but extracted in 0.2 mM EDTA, and lane D was chromatin insoluble in digestion buffer and 0.2 mM EDTA.

Table I: Cleavage and Solubility of Early Histone Gene Chromatin[a]

reaction enzyme	fragment size,[b] n	weight fraction	efficiency of solubilization	efficiency of cleavage, p[c]
*Hin*dIII	$n = 1$	0.24	0.91	0.65
	$n = 2$	0.17	0.83	
	$n \geq 3$	0.60	0.33	
	total		0.65	
*Sal*I	$n = 1$	0.07	0.53	0.39
	$n = 2$	0.09	0.29	
	$n \geq 3$	0.84	0.07	
	total		0.23	
*Sma*I	$n = 1$	0.20	0.36	0.57
	$n = 2$	0.17	0.18	
	$n \geq 3$	0.64	0.09	
	total		0.22	
*Sph*I	$n = 1$	0.15	0.48	0.53
	$n = 2$	0.14	0.34	
	$n \geq 3$	0.71	0.11	
	total		0.28	
*Xho*I	$n = 1$	0.08	0.53	0.25
	$n = 2$	0.12	0.29	
	$n \geq 3$	0.80	0.18	
	total		0.26	

[a] Nuclei were digested at 100 μg of DNA/mL with 5 units of restriction enzyme per microgram of DNA. Digestion buffer was 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, except for *Sma*I buffer which contained KCl instead of NaCl. Soluble chromatin was removed from nuclei by centrifugation according to Materials and Methods. [b] Fragment sizes are given in units of the 6.5-kbp histone repeat. [c] p was calculated for each site on the basis of a statistical cleavage model (see Materials and Methods).

nonspecific endonucleolytic cleavage. As expected, the single-cutting enzymes solubilized monomers, dimers, trimers, and tetramers. Very little additional histone gene chromatin was extracted by the 0.2 mM EDTA. The nuclear pellets contained very high molecular weight histone fragments. *Bgl*II did not produce soluble histone genes.

To estimate the cutting efficiencies of each enzyme, a second experiment was run, taking care to quantitatively recover all the DNA in the restriction supernatants and pellets. The autoradiogram was scanned, and the peaks were integrated to determine the amounts (by weight) of monomer, dimer, and

Table II: Enrichment of Early Histone Gene Chromatin

fragment size, *n*	gradient fractions	fraction of soluble chromatin[a]			enrichment of histone genes from	
		histone genes	bulk chromatin[b]	bulk chromatin[c]	bulk chromatin[b]	bulk chromatin[c]
1	18, 19	0.10	0.08	0.07	1.2	1.4
2	22, 23	0.11	0.10	0.02	1.1	6.2
≥4	30–32	0.11	0.06	0.01	1.8	13.6

[a] Fractions are determined from the specific activities of total chromatin and histone genes (see Figure 6). ^3H counts were used to quantitate chromatin, and ^{32}P counts in dot blots (as described under Materials and Methods) were used to quantitate histone genes. [b] Bulk chromatin after digestion with *Hin*dIII. [c] Bulk chromatin after digestion with *Hin*dIII and *Bgl*II (a null cutter). Enrichment is projected, since the double-digest profile came from a separate gradient. *Bgl*II does not cut the histone gene repeat (see Figure 4) or alter the position of histone gene fragments on the gradients (compare Figures 6 and 5).

higher oligomers of the repeat unit. Table I presents an analysis of the results. For each of these digestions, the majority of the histone genes were present as multimeric species. In every case, the monomers were more soluble than the dimers, the dimers more soluble than the trimers, etc. *Hin*dIII consistently gave the highest fraction of monomer fragments and also gave the highest solubility for fragments of a given size. The correlation between *Hin*dIII sensitivity and fragment solubility might have been due to steric hindrance effects (i.e., insoluble high molecular weight fragments could reduce the diffusion coefficients of shorter fragments) or due to some feature of the nuclear matrix. The fractions of histone genes released correlate well with the fractions of bulk chromatin released during restriction digestion. The site-cutting efficiencies and solubilities that we report are larger than those previously achieved (using high magnesium concentrations) except in the case of short fragments, where solubility might be magnesium concentration independent.

Enrichment for Histone Gene Chromatin Fragments on Glycerol Gradients. The sequence specificity of restriction endonuclease cleavage of chromatin ensures that any fractionation of the chromatin by size will necessarily effect fractionation by sequence. When a specific gene fragment is considerably larger or smaller than the bulk of the soluble chromatin, size fractionation will lead to dramatic enrichment of the gene.

The sea urchin early histone genes provide a good opportunity to test sequence fractionation of chromatin on a glycerol gradient. Hopefully, resolution of multimers of the repeat unit can be achieved. Subsequent to glycerol gradient centrifugation of restricted sea urchin chromatin, dot-blot hybridization of the gradient fractions to pSp102 was performed. ^{32}P was determined by liquid scintillation counting. The gradient profile of histone gene chromatin is shown in Figure 5a. There are two distinct peaks near the center of the gradient followed by a higher molecular weight hump and finally some material at the bottom of the gradient.

We have run a 0.7% agarose–TBE gel on several fractions from this gradient and subsequently blotted and probed the DNA for histone genes. The Southern blot of this gel is shown in Figure 5b. The distribution of histone gene restriction chromatin fragments before centrifugation is shown in the left-most track. The gel lanes show which gradient fractions are enriched in histone repeat monomers, dimers, etc. The slowest sedimenting two gradient peaks clearly represent the monomer and dimer size fragments. Below the dimer peak, the trimers appear followed by tetramers and higher oligomers. The histone genes at the bottom of the gradient consist of an assortment of monomers, dimers, trimers, and higher oligomers. Perhaps some of the chromatin aggregated or was attached to nuclear membrane or matrix.

The histone gene repeat chromatin fragments can be enriched from the bulk chromatin, as shown in Figure 6. This *Hin*dIII digest produced more monomeric histone gene fragments than did the *Sal*I digest, shown in Figure 5, due to the higher cutting efficiency. The distribution of total chromatin in the *Hin*dIII digestion and from a double *Hin*dIII + *Bgl*II digestion is also shown. The digestion with *Bgl*II does not affect the histone gene profile but is intended to reduce the average molecular weight of the non-histone genes.

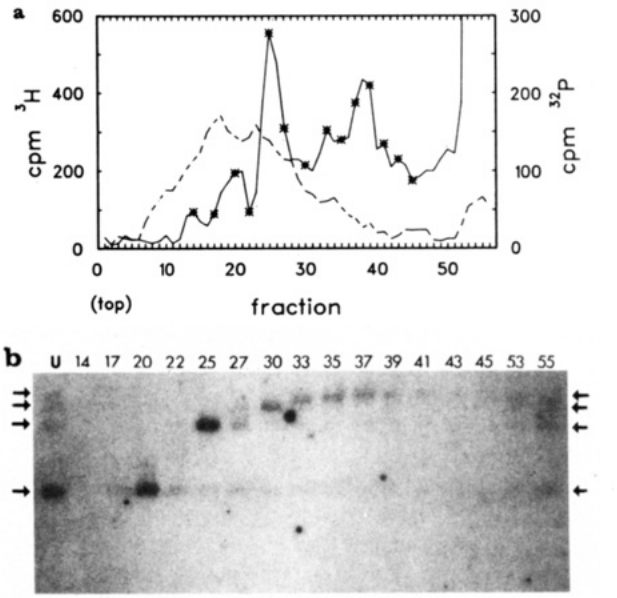

FIGURE 5: Separation of multimers of the 6.5-kbp early histone gene repeat chromatin on glycerol gradients. Panel a shows the distribution of *Sal*I/*Bgl*II-digested [^3H]Leu total chromatin (dashed line) and histone gene chromatin (solid line, by dot blotting and probing with ^{32}P-pSp102) down the gradient (left to right). Panel b shows a Southern blot of selected gradient fractions (shown as stars in panel a). The lanes are numbered according to the fraction numbers. Unfractionated *Sal*I/*Bgl*II-digested chromatin was run in lane U. The repeat monomer peaks in fraction 20, the dimer peaks in fraction 25, the tetramers and higher oligomers peak in fraction 39, and a mixture of all multimers is found near the bottom of the gradient. The locations of monomers, dimers, trimers, tetramers, and larger oligomers are marked by arrows.

The enrichment of histone genes (Table II) was clearly dependent on the molecular weight of the fragments. The monomer peak, which is near the average molecular weight of the chromatin from both the single and double digest, is only slightly enriched. The tetramer and higher peak, which sedimented faster than bulk chromatin, was about 2-fold enriched in the *Hin*dIII experiment and would be up to 16-fold enriched in the double digest. The values in Table II were calculated from the relative amounts of histone genes and total chromatin in each fraction. Digestion by *Bgl*II and *Hin*dIII led to increased enrichment of the high molecular weight histone genes. Simultaneous digestion with *Bgl*II and *Hin*dIII also releases chromatin that would not be released by *Hin*dIII alone, thus increasing the amount of total background chromatin. This could have been avoided by first releasing chromatin with a single cutter and then cutting the soluble chro-

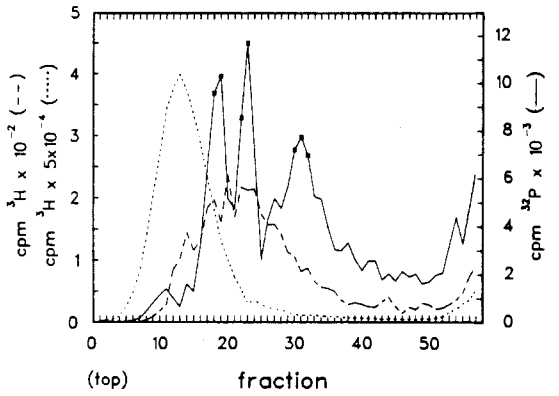

FIGURE 6: Enrichment of histone gene chromatin on glycerol gradients. 12 μg of *Hin*dIII-solubilized sea urchin chromatin was centrifuged as described under Materials and Methods. 40 μL of each fraction was counted to determine the distribution of bulk chromatin (dashed line; specific activity 1900 [^{3}H]leucine cpm/μg of DNA). The histone genes were localized within the gradient by dot hybridization of the remaining 200 μL of each gradient fraction (solid line; specific activity 5.7×10^6 ^{32}P cpm/μg of histone gene DNA). The gradient fractions used for the enrichment calculations in Table II are marked with dots. Also shown are the counts from 40-μL aliquots across a different gradient of sea urchin chromatin digested with *Hin*dIII and *Bgl*II (dotted line; specific activity 114000 [^{3}H]thymidine cpm/μg of DNA). Obviously, the best enrichment would be possible after the double digestion.

matin with *Bgl*II. This method would further enhance the enrichment of histone gene chromatin.

Effects of Restriction Endonuclease Digestion and Glycerol Gradient Fractionation upon Chromosomal Proteins. As an integral part of a separate study, we have carried out extensive controls in order to determine whether chromosomal proteins were proteolyzed, lost, or exchanged during restriction at low concentrations of magnesium or fractionation on glycerol gradients (Workman & Langmore, 1985). Samples of radioactively labeled SV40 minichromosomes and sea urchin embryo chromatin were treated in various ways and then subjected to SDS–PAGE. The proteins were quantitated by fluorography and densitometric analyses.

Chromatin solubilized by restriction enzymes at low magnesium concentrations had the same histone composition as the intact nuclei. Electron microscopy of the restricted SV40 and sea urchin chromatin showed that restriction did not alter the appearance or frequency of nucleosomes on the chromatin fibers.

Successive centrifugations of the SV40 and sea urchin chromatin on glycerol gradients failed to alter the sedimentation coefficients of the molecules. The histone compositions of the chromatin samples were also unaltered by gradient centrifugation. The stoichiometry of non-histone proteins was slightly changed by gradient centrifugation; however, those changes were often less significant than the changes caused by exclusion of the chromatin from Sepharose 4B. In addition, restriction digestion and exposure of the chromatin to high concentrations of glycerol or sucrose did not cause histone exchange to take place.

These results indicate that the genes that we have enriched by restriction and glycerol centrifugation are intact by several criteria.

Discussion

Cleavage and Solubility of Chromatin Depend upon Magnesium Concentration. Low-angle X-ray scattering, electron microscopy, and phase-contrast light microscopy of nuclei have shown that the 30-nm chromatin fibers in 100 mM monovalent salt are much less tightly packed when the magnesium concentration is kept below about 1 mM (Langmore & Paulson, 1983). These observations led us to investigate whether restriction endonucleases would be best able to cleave and solubilize chromatin at low concentrations of magnesium. Our results show that much more chromatin is solubilized by restriction at 0.5 mM Mg than at the 5–10 mM Mg used by other investigators. Under conditions of low magnesium, restriction enzymes were typically able to solubilize 20–70% of bulk chromatin from nuclei of transcriptionally active (sea urchin embryo) and inactive (chicken erythrocyte) nuclei. The restriction sites within the sea urchin early histone genes were cut with 25–65% efficiency, and the resulting chromatin fragments were solubilized with 20–65% efficiency. The solubilization of the fragments was highly molecular weight and enzyme dependent. *Hin*dIII was able to solubilize over 90% of the monomeric histone repeats but only 30% of the trimer and higher oligomers. Typical solubilities for the 6.5-, 13-, and >19-kbp chromatin fragments were 50%, 30%, and 10%, respectively.

On the basis of our studies, there seem to be several important variables affecting chromatin restriction. We expect that increased solubilization would be brought about by any condition, such as reduced magnesium concentration, that was capable of interfering with the higher order structures of chromatin. There is ample evidence in the literature that this is true. For example, in the context of minichromosome digestion, Liggins et al. (1979) have found that decreasing the monovalent salt concentration stimulates restriction of SV40. It is also expected that the digestion of chromatin with nonspecific endonucleases, such as micrococcal nuclease, would be enhanced at lower concentrations of magnesium. Indeed, Varshavsky et al. (1977) found that the digestion of SV40 by nonspecific endonucleases is sensitive to the state of compaction of the minichromosome. We also expect that the molecular weight of the fragments would be very important to the solubility of other genes. For example, Reynolds et al. (1983) were successful in solubilizing 50–70% of the *Xenopus* 5S gene chromatin in spite of using high concentrations of magnesium. Perhaps due to similar reasons, McGhee et al. (1981) were able to release 50–80% of a 115-bp fragment at the 5′ end of the β-globin gene after restriction at 8 mM Mg.

The increased solubilization of cellular chromatin at low magnesium concentrations is probably due to several expected effects, including increased physical accessibility and increased fragment solubility. An unexpected feature was the apparent increase in the restriction of nonsupercoiled chromatin, evident at least for SV40 minichromosomes. Our observations indicate that it is incorrect to correlate nuclease sensitivities with nucleosomal structure alone but that cleavage and solubilization of chromatin fragments depend upon many factors.

Restriction of Chromatin Is Sequence Specific. We have tested the sequence specificity of the restriction endonucleases under the conditions necessary for efficient cleavage and solubilization of chromatin from nuclei. Cleavage of minichromosomes is seen to plateau in less than 1 min in some cases and is dependent upon the presence of restriction enzyme but is not proportional to the enzyme concentration. Thus, small amounts of nonspecific endonucleases are not responsible for the large amounts of chromatin solubilized. Also, even after a 3-h digestion with several single- and null-cutting restriction enzymes, the early sea urchin histone genes are not degraded at sites other than the restriction sequences, and there is no proteolysis. The excellent sequence specificity in the digestion of sea urchin nuclei is a critical step in our purification of histone gene chromatin by nucleoprotein hybridization.

Specific Sequence Chromatin Fragments Can Be Enriched by Size Fractionation. Enrichment of specific genes has been shown by others. Taking advantage of tandem linkage, several investigators have been able to selectively digest and remove other sequences while keeping the sequence of interest intact as high molecular weight insoluble fragments. The repeats were then solubilized with another enzyme and fractionated on sucrose gradients. This approach has allowed Reynolds et al. (1983) to enrich 5S chromatin 7-fold before fractionation on sucrose gradients, which yielded another 3–4-fold enrichment. Using a similar scheme, Zhang & Horz (1982) were able to apply a 50% pure satellite chromatin fraction to sucrose gradients which further purified the satellite sequences to 70%. Lipchitz & Axel (1976) have achieved a 10-fold enrichment of satellite chromatin using sucrose gradients.

We would like to extend the ability to enrich specific sequence chromatin in order to isolate genes that are neither as highly repeated as satellite and 5S RNA genes nor as tandemly linked. In order to do this, it is necessary to have prior knowledge of the restriction sites surrounding the genes of interest. Southern blots of the chromatin restriction digest can reveal which enzymes cut efficiently and what fraction of the genes can be produced in each size fragment. Judicious use of restriction enzymes that make multiple cuts in close proximity, no cuts within the gene of interest, or only widely spaced cuts should ensure that the sedimentation velocity of the gene of interest would be sufficiently different from the bulk chromatin to achieve excellent enrichment by centrifugation.

We have demonstrated the enrichments possible with chromatin fragments that are multimers of 6.5 kbp after a single digestion or a double digestion including a null-cutting restriction endonuclease. To our knowledge, the 16-fold enrichment of the histone genes that we achieved is the highest enrichment of any gene by gradient fractionation to date. Similar enrichment should be possible with any restriction fragment of comparable size. In the case of the early histone genes, which represent 0.25% of the sea urchin genome (Cohn et al., 1976), the 16-fold enriched fraction is 4% pure.

Usefulness of Size Fractionation in Conjunction with Other Techniques. Although only a few highly repeated genes could ever be purified as chromatin solely by size fractionation, the enrichment of specific genes and their associated proteins on glycerol gradients provides a very useful tool in studies of proteins and histone modifications associated with specific genes. Dot blots of gradient fractions can be used to locate the fractions containing specific genes and to quantitate their enrichment. True gene-specific chromosomal proteins should also be enriched in those fractions. If the sequences are present in high copy number, the proteins could be detected by acylamide gel electrophoresis. If the sequences are rare but antibodies are available to the proteins, the proteins could be detected by dot blotting gradient fractions and probing the filters with antibodies. Gradient fractions could be assayed with DNA probes and antibodies in order to directly compare the sedimentation profiles of the genes and putative gene-specific protein. This approach could be useful for identification of regulatory proteins by in vitro reconstitution (Payvar et al., 1981; Weideli et al., 1980; Emerson & Felsenfeld, 1984; Mulvihill et al., 1982; Gross et al., 1982; Strauss & Varshavsky, 1984). If these putative gene-specific proteins cosediment with chromatin fragments containing those genes, it would provide direct evidence of their role in vivo.

Using the gradient dot-blot method, it is possible to examine some aspects of the higher order structure of specific genes. Chromatin fibers have been shown to have a salt-dependent sedimentation velocity (Butler & Thomas, 1980; Thomas & Butler, 1980; Allan et al., 1981). However, specific genes in certain states of activity may not condense into thick fibers. These sedimentation velocity changes are detectable on glycerol gradients run at different ionic strengths (our unpublished results). Kimura et al. (1983) have already carried out such a study on sucrose gradients using Southern blots and have found that β-globin gene chromatin from chicken erythrocytes does not show an increase in sedimentation velocity with increasing salt.

The efficient restrictions and gradient enrichments described in this paper are also of essential utility in our general program to highly purify specific genes as chromatin (Workman & Langmore, 1985). After prefractionation on glycerol gradients, a technique termed "nucleoprotein hybridization" will be used to enrich specific sequence chromatin fragments over 115-fold without histone loss or exchange. This method utilizes the specific ends of the chromatin restriction fragments. Exonuclease digestion of these ends produces specific sequence single-stranded tails. We have been able to hybridize specific sequence mercurated DNA probes to the tails of those fragments. Subsequent affinity chromatography (using sulfhydryl–Sepharose to bind the mercury) isolates the probe and the hybridized specific sequence chromatin fragments. We have achieved over a hundredfold enrichment of the early histone genes of *S. purpuratus* by the method of nucleoprotein hybridization (unpublished results).

Used in series, gradient fractionation allows us to pre-enrich for histone gene chromatin before subjecting it to nucleoprotein hybridization. This pre-enrichment significantly reduces the expense of processing large amounts of chromatin as well as increases the ultimate purity possible. Assuming that the glycerol gradients can provide 16-fold enrichment of histone genes, and that nucleoprotein hybridization can provide an independent enrichment of 115-fold, it would be possible to enrich sea urchin histone gene chromatin to better than 80% purity. Used in parallel, gradient fractionation provides us with a valuable independent method to confirm that the proteins that are isolated by nucleoprotein hybridization were actually bound to the same DNA sequences before hybridization.

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Registry No. Mg, 7439-95-4; restriction endonuclease, 9075-08-5; restriction endonuclease *Hin*dIII, 81295-22-9; restriction endonuclease *Sal*I, 81295-38-7; restriction endonuclease *Sma*I, 82391-42-2; restriction endonuclease *Sph*I, 85270-15-1; restriction endonuclease *Xho*I, 81295-43-4; restriction endonuclease *Eco*RI, 80498-17-5; restriction endonuclease *Kpn*I, 81295-27-4; restriction endonuclease *Bgl*II, 81295-12-7.

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