Preparation of soluble chromatin and specific chromatin fractions with restriction nucleases

---

Tibor Igo-Kemenes, Walter Greil, and Hans G. Zachau

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, D-8000 München 2, GFR

Received 30 August 1977

ABSTRACT

By digestion of rat liver nuclei with EndoR HaeIII, EndoR EcoRI, and EndoR Bam and subsequent lysis of the nuclei approx. 90 %, 40 %, and 45 %, respectively, of the chromatin were solubilized. The plateau values of solubilization are in agreement with a model in which the chromatin strands are crosslinked and/or attached to a supporting structure. The distribution of DNA lengths in the soluble and insoluble chromatin fractions were determined. According to digestion experiments with restriction nucleases rat liver DNA contains highly repetitive sequences, some of which are arranged in tandem repeats of 95 and 380 nucleotide pairs, respectively. With EndoR EcoRI chromatin containing the repetitive DNA was preferentially solubilized and, by subsequent sucrose gradient centrifugation, purified to about 90 %. The useful properties of chromatin prepared by the specific action of restriction nucleases are discussed.

INTRODUCTION

Besides electron microscopy and a number of other physical techniques digestion studies with non-specific nucleases have laid the foundation of our present knowledge of chromatin structure (summaries 1,2). Partial digestion with micrococcal nuclease³ has also replaced, for the preparation of soluble chromatin, the classical methods of chromatin solubilization which involve shearing. For the preparation of specific chromatin fragments restriction nucleases seem to be superior to the non-specific nucleases. We have previously shown that, at high concentrations, EndoR Bsu and EndoR EcoRII can cleave the DNA within mouse and rat liver nuclei⁴. The action of endogenous DNAases could be very largely suppressed so that

cleavage occurred practically only at the restriction sites⁵. In these experiments the restriction nucleases cleaved very predominantly within the linker region between nucleosome cores. EndoR EcoRI has been used by Lipchitz and Axel for digesting calf thymus nuclei and for enriching satellite DNA containing chromatin, whereby extensive cleavage within the nucleosome cores was indicated⁶. We have continued our studies on restriction nuclease cleavage of chromatin, both, in analytical and preparative directions. The detailed analysis of the cleavage patterns led to a "domain model" of chromatin structure in which crosslinking between chromatin strands and/or an attachment to a supporting structure is described⁷. The preparative work with restriction nucleases which is described in this paper, included attempts to prepare a defined soluble chromatin in good yield and to investigate the preferential solubilization of chromatin fractions, containing specific DNA sequences.

MATERIALS AND METHODS

Materials. Seakem agarose for gel electrophoresis was from Marine Colloids Inc., Rockland, Mne; [³H]-thymidine, 26 Ci/mmole, from New England Nuclear, Dreieichenhain, W. Germany; Proteinase K from E. Merck, Darmstadt; micrococcal nuclease and pancreatic ribonuclease from Worthington Biochem. Corp., Freehold, N.J. EndoR EcoRI restriction nuclease was prepared as described⁸ including the phosphocellulose step. One unit is defined as the amount of enzyme, which is sufficient to digest to completion 1 µg SPP1 phage DNA in a total volume of 20 µl at 37° in 1 h. EndoR HaeIII was prepared by R. Streeck from *Haemophilus aegyptius*, obtained from H. Schaller, according to the method of Roberts et al.⁹, and kindly donated. One unit is defined as the amount of enzyme which is sufficient to digest to completion 4 µg of λdv1 plasmid DNA in a total volume of 50 µl at 37° in 3 h. EndoR BamI was prepared by U. Hänggi from *Bacillus amyloliquefaciens* strain H according to ref. 10 and kindly donated. One unit is defined as the amount of enzyme which is sufficient to digest to completion 1 µg λ phage DNA in a total volume of 100 µl at 37° in 1 h.

Preparation of ^3H -thymidine labeled and unlabeled nuclei.

Rats (150-200 g) were partially hepatectomized under ether anesthesia. 30 hours after operation¹¹ 0.2 mCi (^3H)-thymidine were injected intraperitoneally in 1 ml 0.15 M NaCl, 10 mM phosphate buffer pH 7. The rats were sacrificed one or two days later. Nuclei from normal or regenerating rat liver were prepared as described¹² and kept frozen as a moist pellet at -80° for several months.

Digestion of nuclei with restriction nucleases. Aliquots of frozen nuclei were suspended by gentle homogenization in buffer A (65 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 5 mM β -mercaptoethanol, 15 mM Tris-HCl, pH 7.4) and washed once or twice at 0° . Finally nuclei containing about 1.2 mg DNA were dispersed in a total volume of 200 μl buffer A, containing 10 mM MgCl_2 (unless otherwise stated) together with the amounts of restriction nucleases indicated in the legends. It should be noted that all incubation mixtures contained glycerol (up to 15 %) and those with EndoR EcoRI also Triton X-100 (up to 0.02 %). These components which were contained in the storage buffers of the restriction nucleases had no detectable effect in the digestion experiments. Digestions were started by bringing the mixtures to 37° . In kinetic experiments, 20 μl aliquots were taken and the reaction terminated by the addition of 480 μl icecold buffer A containing 5 mM EDTA, 0.5 mM EGTA, and 0.2 % Triton X-100, unless otherwise stated. For every time point aliquots were withdrawn in order to determine the total amount of DNA. Other aliquots were saved for the determination of the DNA size distribution of the (not yet fractionated) chromatin. Nuclei were then pelleted gently by centrifugation at $2000 \times g$ for 1-2 minutes and washed again in the same buffer but without Triton X-100. The combined supernatants were analyzed for DNA, RNA, and protein contents⁷.

Lysis of nuclei and chromatin extraction. The pelleted and washed nuclei were rapidly mixed with 1.0 ml 0.2 mM EDTA, adjusted to pH 7.9 with Tris base. Extraction of soluble chromatin was performed at 0° under gentle agitation for at least 5 h or overnight. When the nuclei were completely lysed and dispersed in the extraction fluid, the suspension was

centrifuged at 10 000 x g at 4° for 15 min. The insoluble chromatin pelleted as a gel, while the soluble chromatin fraction was present in the clear supernatant. If necessary, the extraction was repeated once more for 3-5 h. The amount of chromatin present in the various fractions was determined from their DNA content, either by measuring the optical densities at 260 nm, or by determination of their radioactivity. Radioactivity measurements were done by liquid scintillation counting after combustion of aliquots in a sample oxidizer (Oxymat, Deutsche Intertechnique, Mainz). Quench correction was by the external standard technique.

Analysis of DNA chain length distribution by gel electrophoresis. DNA was prepared by treatment of chromatin with 100-200 µg/ml Proteinase K in 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 0.1 % SDS. Extractions were omitted to avoid shearing of the DNA. DNA derived from the soluble chromatin fractions was concentrated after Proteinase K treatment by a very careful ethanol precipitation, and subsequent dissolution in 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA. This treatment did not shorten the chain lengths of T5 and T4 phage DNA. About 0.05-0.15 A₂₆₀ units of DNA were applied to 0.3 % vertical agarose slab gels in 36 mM Tris, 30 mM KH₂PO₄, 1 mM EDTA and run at 0.8 V/cm for about 10 h. Electrophoreses of DNA samples on 0.8-1.5 % agarose slab gels and preparative electrophoreses were as described¹². DNA was visualized in the gels and photographed¹². Molecular weight markers were DNA fragments of 44, 30, 25, 14, 3.2, and 2.4 kb from EndoR EcoRI digested wild type T5 phage DNA¹³, of 6.5 and 0.5 kb of EndoR HindIII digested λdv1 plasmid DNA¹⁴, 1670, 1307, 877, 516, 447, 344, 258, 214, 205, 172, and 142 nucleotide pairs of EndoR HaeIII digested λdv1 DNA¹⁵. In addition a partial EndoR EcoRII digest of mouse satellite DNA was used with 242 nucleotide pairs and multiples thereof. The fragments were kindly donated by G. Peter, M. Steinmetz, and W. Hörz.

RESULTS AND DISCUSSION

Preparation of soluble chromatin from rat liver nuclei with EndoR HaeIII, EndoR EcoRI, and EndoR Bam I. An important

point in our work was that endogenous DNAases, but not necessarily RNAases, can be largely suppressed when the nuclei were prepared and washed in a spermine-spermidine containing buffer. In our initial experiments a buffer with 80 mM salt was used⁴ corresponding to the buffer originally used by Hewish and Burgoyne¹⁶. We later altered the conditions to 150 mM salt (ref. 5 and the present study) in order to work in the range of minimal solubility of chromatin^{17,18}. When nuclei were incubated in this buffer with restriction nucleases, washed and centrifuged about 98 % of the chromatin remained insoluble. Only about 2 % of the chromatin were released into the supernatant together with some nuclear RNA and non-histone proteins; the DNA in this fraction was of small size and histone H1 was largely absent. While the use of detergents was avoided during the preparation of the nuclei and, as much as possible, during digestion with restriction nucleases, Triton X-100 was added to the wash fluid in order to obtain a high recovery of nuclei. The actual solubilization of chromatin was effected by suspending the washed nuclei in 0.2 mM EDTA according to ref. 3; pH 7.9 was used instead of a neutral pH in order to achieve maximal solubilization. The presence of 10 mM Tris did not influence the efficiency of extraction, while 100 mM Tris or 50 mM Tris plus 20 mM NaCl led to a lower efficiency of extraction. In the presence of 2 mM CaCl_2 practically no chromatin was solubilized. The time required for extraction of chromatin depended on a number of factors as surface ratio etc. Clearly chromatin with long DNA was extracted more slowly than short chromatin pieces.

With EndoR HaeIII 87-92 % of the chromatin was solubilized (Fig. 1). There was a reasonable correlation between amount of nuclease and time of incubation; in the absence of nuclease practically no chromatin was solubilized. The remaining 8-13 % of chromatin could also not be solubilized in the presence of up to 2 % Triton X-100 indicating that phospholipids are not the major cause of its insolubility. Solubilization in the presence of 0.6-2.0 M NaCl was possible but led to a chromatin in which histone H1 was removed and the nucleosome cores had migrated or were dissociated¹⁹.

Figure 1. Solubilization of rat liver chromatin with EndoR HaeIII. Digestion was done with 200 (●—●), 400 (▼—▼), 800 units (■—■) and without nuclease (○—○). The increase of the optical density at 260 nm was taken as a measure for chromatin solubilization. Triton X-100, which would have interfered with A_{260} -measurements, was omitted from all buffers in these experiments.

The distribution of DNA lengths in the soluble and insoluble chromatin fractions was always rather broad (Fig. 2). At early times of digestion the DNA in the pelleted chromatin was considerably bigger than in the soluble fraction. At later times the differences disappeared and a chromatin with a weight average DNA chain length of 2 650 nucleotide pairs was formed. Fig. 3 shows that chromatin solubilized with EndoR HaeIII possessed the normal nucleosome structure and that the histone composition was indistinguishable in the soluble fraction and in intact nuclei.

With EndoR EcoRI approx. 40 % of the chromatin were solubilized (Fig. 4a). Addition of more nuclease did not lead to a higher plateau value. In the absence of nuclease or Mg^{++} practically no solubilization of chromatin took place. An experiment with EndoR BamI (Fig. 4b) is included in order to show that the situation with respect to this restriction nuclease was rather similar to the one with EndoR EcoRI. No definitive statement can be made yet, however, as to the plateau level since our EndoR BamI preparations contained a

Figure 2. EndoR HaeIII cleavage of rat liver nuclei. Analysis of DNA size distribution from soluble (—) and insoluble (---) chromatin on a 0.35% agarose slab gel in the Tris-borate buffer system²⁰ with 1 μ g/ml ethidium bromide. The chromatin fractions were derived from the kinetic experiment described in Fig. 1, using 200 units of enzyme. A, 10 min digest. The molecular weight calibration curve was obtained using an EndoR EcoRI digest of T5 DNA (■—■), EndoR HaeIII digest of λ dv1 (▲—▲), and a partial EndoR EcoRII digest of mouse satellite DNA (●—●). B, 3 h digest of Fig. 1.

small amount of contaminating nuclease(s) which became significant at the high restriction nuclease concentrations required for chromatin solubilization.

Preferential solubilization of chromatin fractions containing highly repetitive DNA. In previous work of this laboratory it was shown that rat DNA contains highly repetitive component(s) which were not separated from the rest of the DNA but which on digestion with the EndoR Hind nucleases and EndoR EcoRI yielded in gel electrophoretic separations series of sharp bands²¹. Similar studies with EndoR Bsu were reported recently⁵. Highly repetitive sequences in rat DNA have also been detected by other authors²²⁻²⁴. Since frequent cleavage by restriction nuclease in the repetitive components should

Figure 3. Nucleosomal DNA patterns and histone composition of soluble chromatin. A. EndoR HaeIII solubilized DNA was submitted to gel electrophoresis (a); another aliquot of 1 ml with about 0.8 A₂₆₀ units was adjusted with Tris-HCl, pH 7.6, and CaCl₂ to 20 mM and 2 mM, respectively, and digested with 1 unit micrococcal nuclease for 5 min at 37° (b). The reaction was terminated by the addition of 5 mM EDTA. The DNA was run on a 1.5 % agarose slab gel. B. Histones were extracted from unincubated nuclei (d), from nuclei incubated for 1.5 h with 400 units EndoR HaeIII (c), from the combined first supernatants (b) and from soluble chromatin of the same incubation (a). SDS-polyacrylamide gels were run as in ref. 5.

lead to a preferential solubilization of the respective chromatin, these components were investigated in some detail. EndoR Alu and, less pronounced, EndoR EcoRII produced series of bands from rat DNA (W. Pfeiffer, unpubl.) while EndoR BamI seemed to produce only or mainly one band corresponding to DNA of 3.4×10^6 dalton. In limit digests of rat liver DNA with EndoR EcoRI strong bands were seen of 360, 440, 1450, and 2425 nucleotide pairs as well as the mono- to tetramer of a 95 ± 1 (standard deviation) nucleotide pair fragment (Fig. 5b). In partial digests the regular ladder extended at least to the 25-mer of the 95 nucleotide pair fragment and additional weak and strong bands appeared (Fig. 5c). Higher members of the regular series yielded mono- to pentamers on complete digestion with

Figure 4. Solubilization of rat liver chromatin with EndoR EcoRI and EndoR BamI. A. Digestion was with 35 000 units EndoR EcoRI (●—●), with the same amount of nuclease but without Mg^{++} (○—○), or without nuclease (○—○). B. Digestion was with 3 000 units EndoR BamI (●—●) or without nuclease (○—○). Digestion times were as indicated. Solubilization was measured by determination of the radioactivity in the fractions.

EndoR EcoRI (Fig. 5d-g), while the 1450 nucleotide pair DNA could be further digested only with other restriction nucleases as EndoR Bsu and EndoR EcoRII. In EndoR HindIII digestions of rat DNA, fragments of a 380 ± 4 nucleotide pair repeat were found, up to the tetramer in limit digests and up to the octamer in partial digests (Fig. 5h,i). From the digestion of the 380 nucleotide pair EndoR HindIII fragment with EndoR EcoRI (Fig. 5l,m) it can be concluded that the cleavage sites for the two nucleases are located on the same repetitive component and that the stagger of the cleavage sites is 38 or 57 nucleotide pairs, respectively.

In the experiments on chromatin solubilization we concentrated on EndoR EcoRI. It is obvious from the experiment shown in Fig. 6 that the highly repetitive DNA was considerably enriched in the soluble chromatin fraction; equal amounts of the soluble and insoluble fraction were applied to the respective slots. An exact determination of the degree of enrichment was difficult but on the basis of the scans an

Figure 5. Digestion of total DNA and DNA fragments from rat liver with restriction nucleases. DNA was digested in the same buffer as chromatin with an amount of EndoR EcoRI necessary to carry the reaction to completion at 37° within 2 h. 0.15 A₂₆₀ units of the 2.5 min partial digest (b) and of a 2 h limit digest (c) were applied. The octamer (d) and the undecamer (f) of the 95 nucleotide pair unit was isolated on preparative gels and rerun. Further digestion of the octamer (e) and the undecamer (g) with EndoR EcoRI. DNA was digested with the amount of EndoR HindIII necessary to produce a limit digest (i) within 140 min. The 40 min partial digest is shown (h). The 380 nucleotide pair band from the EndoR HindIII digest was isolated on preparative gels and rerun (l), or further digested with EndoR EcoRI (m). (a,k) are marker fragments from EndoR HaeIII digested λ dv1 DNA. a-c was run on a 0.8 %, d-g on a 1.5 %, h-k on a 1 %, and l,m on a 1.5 % agarose gel.

about fourfold enrichment of repetitive sequences in the soluble fraction compared to the unfractionated chromatin could be estimated. A further purification was achieved by sucrose gradient centrifugation of the soluble fraction (Fig 7A). The most slowly sedimenting chromatin fraction contained very largely repetitive DNA (Fig. 7B). On the basis of scans a purity of

Figure 6. Preferential solubilization of repetitive DNA containing chromatin with EndoR EcoRI. Rat liver nuclei were digested with EndoR EcoRI to completion. DNA was prepared from nuclei (b), from soluble chromatin (c) and insoluble chromatin (d), and separated on a 0.8 % agarose slab gel. About 10 μ g of DNA was applied to each slot. A partial EndoR EcoRII digest of mouse satellite DNA was included (a) as marker DNA; the numbers denote multiples of the 242 nucleotide pair fragment.

about 90 % could be estimated.

The fastest moving bands in electrophoretic separations of DNA from EndoR EcoRI solubilized chromatin always corresponded to the 380 nucleotide pair DNA, also in late time points of digestion. This can be seen in Fig.6 and more clearly in Fig.7B, track (a) where the starting material of the sucrose gradient experiment is shown. When this chromatin fraction was deproteinized and again digested with EndoR EcoRI the mono- to tetramers of the 95 nucleotide pair series were formed, track (b). Apparently in the repetitive DNA containing chromatin cleavage by EndoR EcoRI was suppressed not only at distances of 95 and 285 nucleotide pairs (which should lie within nucleosomes in

Figure 7. Enrichment of repetitive DNA containing chromatin on sucrose gradients. Soluble chromatin was formed by complete digestion of nuclei with EndoR EcoRI. 3 A_{260} units each were layered on isokinetic sucrose gradients ($C = 5\%$, particle density 1.51, ref. 25) and centrifuged in a SW 41 rotor of a Beckman Spinco ultracentrifuge at 4° for 4 h at 40 000 rpm. The scan of one of the gradients is shown in panel A. Fractions from four tubes were pooled as indicated and deproteinized. Samples of 2–5 μ g DNA each were separated on 0.8 % agarose gels, panel B. a, Chromatin before gradient centrifugation; b, same material as before but the DNA was digested to completion with EndoR EcoRI; c–f, pools 1–4 of the sucrose gradient.

any case) but also at the 190 nucleotide pair distance. The occurrence of the 190 nucleotide pair fragment would be expected if the usual nucleosome arrangement with 140 and 60 nucleotide pairs in the core and linker regions, respectively, were to apply also to the repetitive DNA containing chromatin. It cannot be excluded that the absence of the band reflects some special feature of EndoR EcoRI cleavages, but most likely it will give clues as to the structure of this chromatin fraction.

CONCLUDING REMARKS

High concentrations of restriction nucleases are required in order to digest in a few hours chromatin within nuclei to completion as was found previously in our laboratory^{4,5} and was confirmed in the present work. Considering the high concentration of DNA in the nucleus this is not surprising. In spite of the high nuclease concentrations cleavage seems to occur, according to statistical analysis⁷, predominantly in the linker regions between nucleosome cores. The observation of Lipchitz and Axel⁶ of cleavage by EndoR EcoRI within the nucleosome cores of calf thymus nuclei may be related to species differences or differences in the preparation of the nuclei. The finding of plateau values in the solubilization of chromatin after restriction nuclease digestion is in agreement with the domain model of chromatin structure⁷. The preferential solubilization of repetitive DNA containing chromatin demonstrates the usefulness of the approach. In future, the distribution of coding sequences in chromatin fractions prepared with different restriction nucleases will be investigated.

ACKNOWLEDGEMENT

We are indebted to I. Brenner for able technical assistance. The work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

- 1 Elgin, S.C.R., and Weintraub, H. (1975) *Annu. Rev. Biochem.* 44, 725-774.
- 2 Kornberg, R. (1977) *Annu. Rev. Biochem.* 46, 931-954.
- 3 Noll, M., Thomas, J.O., and Kornberg, R. (1975) *Science* 187, 1203-1206.

- 4 Pfeiffer, W., Hörz, W., Igo-Kemenes, T., and Zachau, H.G. (1975) *Nature* 258, 450-452.
- 5 Hörz, W., Igo-Kemenes, T., Pfeiffer, W., and Zachau, H.G. (1976) *Nucl. Acids Res.* 3, 3213-3226.
- 6 Lipchitz, L., and Axel, R. (1976) *Cell* 9, 355-364.
- 7 Igo-Kemenes, T., and Zachau, H.G. (1977) Cold Spring Harbor Symp. Quant. Biol. 42 in press.
- 8 Greene, P.J., Betlach, M.C., Boyer, H.W., and Goodman, H.M. (1974) *Methods Mol. Biol.* 7, 87-111.
- 9 Roberts, R.J., Breitmeyer, J.B., Tabachnik, N.F., and Myers, P.A. (1975) *J. Mol. Biol.* 91, 121-123.
- 10 Wilson G.A., and Young, F.E. (1975) *J. Mol. Biol.* 97, 123-125.
- 11 Takatsuka, Y., Kohno, M., Higashi, K., Hirano, H., and Sakamoto, Y. (1976) *Exp. Cell Res.* 103, 191-199.
- 12 Greil, W., Igo-Kemenes, T., and Zachau, H.G. (1976) *Nucl. Acids Res.* 3, 2633-2644.
- 13 Rhoades, M. (1975) *Virology* 64, 170-179.
- 14 Streeck, R.E., and Hobom, G. (1975) *Eur. J. Biochem.* 57, 595-606.
- 15 Streeck, R.E., and Zachau, H.G. (1977) manuscript in preparation.
- 16 Hewish, D.R., and Burgoyne, L.A. (1973) *Biochem. Biophys. Res. Commun.* 52, 504-510.
- 17 Leake, R.E., Trench, M.E., and Barry, J.M. (1972) *Exp. Cell Res.* 71, 17-26.
- 18 Bradbury, E.M., Danby, S.E., Rattle, H.W.E., and Giancotti, V. (1975) *Eur. J. Biochem.* 57, 97-105.
- 19 Steinmetz, M., Streeck, R.E., and Zachau, H.G. (1977) *Eur. J. Biochem.* submitted
- 20 Peacock, A.C., and Dingman, C.W. (1967) *Biochemistry* 6, 1818-1827.
- 21 Philippsen, P., Streeck, R.E., and Zachau, H.G. (1974) *Eur. J. Biochem.* 45, 479-488.
- 22 Roizes, G. (1974) *Nucl. Acids Res.* 1, 1099-1120.
- 23 Pays, E., and Ronsse, A. (1975) *Biochem. Biophys. Res. Commun.* 62, 862-867.
- 24 Guttann, T., Votavova, H., and Pivec, L. (1976) *Nucl. Acids Res.* 3, 835-845.
- 25 McCarty, K.S., Jr., Vollmer, R.T., and McCarty, K.S. (1974) *Analyt. Biochem.* 61, 165-183.