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Analysis of highly purified satellite DNA containing chromatin from the mouse

Xian Yang Zhang and Wolfram Hörz

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, München, FRG

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ABSTRACT

A purification scheme for satellite DNA containing chromatin from mouse liver has been developed. It is based on the highly condensed state of the satellite chromatin and also takes advantage of its resistance to digestion by certain restriction nucleases. Nuclei are first treated with micrococcal nuclease and the satellite chromatin enriched 3-5 fold by extraction of the digested nuclei under appropriate conditions. Further purification is achieved by digestion of the chromatin with a restriction nuclease that leaves satellite DNA largely intact but degrades non-satellite DNA extensively. In subsequent sucrose gradient centrifugation the rapidly sedimenting chromatin contains more than 70 % satellite DNA. This material has the same histone composition as bulk chromatin. No significant differences were detected in an analysis of minor histone variants. Non-histone proteins are present only in very low amounts in the satellite chromatin fraction, notably the HMG proteins are strongly depleted.

INTRODUCTION

Constitutive heterochromatin was originally detected by cytological methods and defined as that part of the chromosomal structure that does not decondense during interphase (1,2). The heterochromatic DNA consists in its majority of highly repetitive sequences (3) which are presumed to be genetically inactive. Chromatin that contains satellite DNA which, for reasons of brevity, we will refer to as satellite chromatin, has been analyzed in a variety of organisms by biochemical means (reviewed in ref. 4) but the molecular basis for the distinct appearance of heterochromatin is not known. In all likelihood chromatin components other than the repetitive DNA itself are involved since euchromatic segments artificially relocated next to heterochromatin can be repressed in their function, an effect which is

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known as position variegation (5).

We have previously shown for satellite chromatin from mouse liver nuclei that its characteristically condensed state is highly sensitive to the ionic environment (6). These studies have now been extended and a purification scheme for mouse satellite chromatin was developed. The procedure is based on the higher compaction of satellite chromatin as well as its larger size after digestion of chromatin by restriction nucleases.

The conditions used for the preparation of satellite chromatin are very gentle. There are no mechanical shearing steps involved and buffers of extreme pH, high concentrations of salt or urea are avoided. The resulting material contains high molecular weight DNA and is amenable to various analyses.

MATERIALS AND METHODS

Preparation of nuclei. Nuclei from mouse liver were isolated as described (7) with the inclusion of 0.1-0.5 mM PMSF and 15 mM β-mercaptoethanol in all buffers. For the preparation of ^{14}C-thymidine labeled nuclei, mice (20-30 g) were partially hepatectomized under ether anesthesia. After 30 h (8) 15 µC ^{14}C-thymidine per mouse were injected intraperitoneally and 24 h later a second injection of 5 µC ^{14}C-thymidine was given. The mice were sacrificed one day later (9), and the nuclei isolated.

Digestion of nuclei with micrococcal nuclease and Bsp and solubilization of chromatin. Bsp (10) was prepared according to ref. (11). Digestion with micrococcal nuclease was carried out at 37^{o} as described (7), and with Bsp under the same conditions except that the buffer was supplemented with 10 mM $MgCl_2$ instead of 1.4 mM $CaCl_2$. The concentration of the nuclei during digestion with micrococcal nuclease was 0.25 mg DNA/ml and for Bsp digestion 2-5 mg DNA/ml. After digestion the chromatin was centrifuged at 5000 g for 10 min extracted with different buffers and again subjected to centrifugation at 5000 g for 10 min. Solubility of the chromatin was either determined by radioactivity measurements or by measuring the optical density of the DNA isolated from different chromatin fractions.

DNA analysis by gel electrophoresis and analytical ultracentrifugation. DNAs from different fractions were isolated by

proteinase K treatment and analyzed by agarose gel electrophoresis as described (7). Analytical ultracentrifugation of DNA in CsCl was carried out in the presence of the Hoechst dye 33258 as described (12,6). The satellite DNA content was determined from the relative peak areas of the scans.

Protein analyses. Chromatin fractions were lyophilized, dissolved in sample buffer, heated to 100° for 10 min and total proteins analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to Laemmli (13). The separating gel contained 18 % acrylamide and 0.12 % bisacrylamide.

High mobility group (HMG) proteins were prepared according to Goodwin et al. (14). Nuclei or chromatin fractions were extracted with 0.35 M NaCl and the extract was made 2 % in TCA. The HMG proteins, which remain soluble under these conditions, were precipitated with acetone and analyzed in 20 % acetic acid/urea polyacrylamide slab gels (32).

Histones were extracted from chromatin with 0.25 N HCl and precipitated with acetone (15) or lyophilized. In order to resolve the H1 histones, a 12 % polyacrylamide gel (40 cm long) containing 6 M urea and 5 % acetic acid (16) was used. Core histone variants were visualized in a 12.5 % polyacrylamide gel containing 7.5 M urea, 5 % acetic acid, and 0.3 mM Triton-X 100 (17). The urea containing gels were stained with Coomassie Blue-R 250 and scanned at 600 nm (24) while SDS/polyacrylamide gels were stained with the silver method according to Merril et al. (18) as modified by R.M. Tanguay (personal communication).

RESULTS

Enrichment of satellite chromatin after digestion of nuclei with micrococcal nuclease. The procedure we have previously reported for the enrichment of mouse satellite chromatin (6) relies on its higher compaction in the presence of Ca^{++} ions as compared to bulk chromatin. This difference is highly sensitive to the ionic strength, and it is destroyed by EDTA or monovalent ions at concentrations of more than 5 mM. In an effort to extend the purification scheme for satellite chromatin we looked more closely at the solubility properties of satellite

chromatin and bulk chromatin. Mouse liver nuclei were digested with micrococcal nuclease, the chromatin washed three times in 10 mM Tris-HCl, pH 7.4, 3.3 mM $CaCl_2$, and then extracted in a variety of low ionic strength buffers. Total solubility and satellite DNA content of the insoluble fraction are shown in Fig. 1. It is evident that some salt is required for chromatin solubilization. At low concentrations of monovalent ions it is almost exclusively bulk chromatin which is solubilized, and satellite chromatin is correspondingly enriched in the insoluble fraction. The content of satellite DNA reaches 30-40 % in the pellet fraction when 1 mM Tris-HCl or 0.5 mM Tris-HCl, 0.5 mM NaCl are used. At higher Tris-HCl or NaCl concentrations satellite chromatin also begins to be solubilized. Low concentrations of EDTA solubilize essentially all of the chromatin even in the absence of monovalent ions.

The enrichment of satellite chromatin is dependent on the Ca^{++} washing of the chromatin as just described. If this step is omitted, about 80 % of the chromatin can be solubilized in 0.2 mM Tris-HCl, pH 8.0, with no enrichment of satellite DNA in the pellet fraction. This is the case even though Ca^{++} is al-

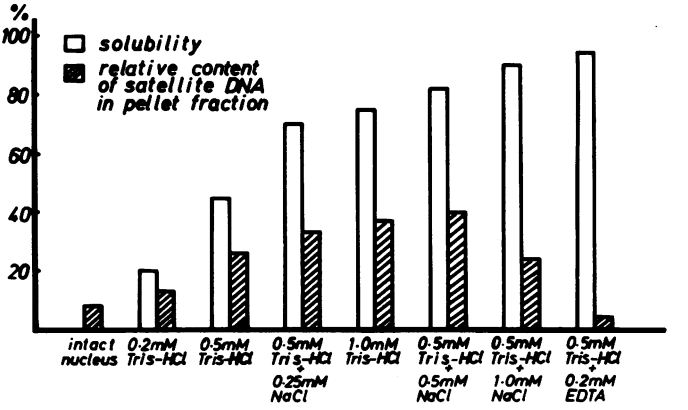

Figure 1. Differences in the solubility of bulk chromatin and satellite chromatin. Mouse liver nuclei labeled with ^{14}C-thymidine were digested with micrococcal nuclease (3 U/ml, 20 min), washed three times with 3.3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, and then extracted with different buffers. Solubility of total chromatin was determined by radioactivity measurements and the satellite DNA content by analytical ultracentrifugation in CsCl.

ways present during digestion with micrococcal nuclease. Interestingly, Ca^{++} treatment is ineffective if the chromatin is first exposed to low ionic strength buffer and only then washed with Ca^{++}. No enrichment of satellite DNA is observed in subsequent fractionation schemes under these conditions.

A two step procedure for the isolation of satellite chromatin. For the further purification of satellite chromatin a pellet fraction enriched for satellite DNA after micrococcal nuclease digestion as described in the preceding paragraph was further digested with Bsp, a restriction nuclease which leaves satellite DNA largely intact but degrades main band DNA extensively (19). As a consequence of this it should be possible to separate satellite DNA chromatin by virtue of its larger size from bulk chromatin. The extent of digestion by micrococcal nuclease in the first step was kept low which decreased the amount of soluble chromatin to 70 % and correspondingly diminished the percentage of satellite DNA in the pellet fraction to 25 %. This way, however, the material in the pellet fraction remained of high enough molecular weight to be suitable in the subsequent fractionation according to size.

As shown in Fig. 2a the chromatin remaining insoluble after micrococcal nuclease digestion was further degraded with Bsp and afterwards extracted first with 1 mM Tris-HCl, and then with 10 mM Tris-HCl. In the second extraction step about 30 % of the Bsp digested chromatin could be solubilized (equivalent to 10 % of the original nuclear material). Approximately 50 % of the DNA in this fraction (S_{B_2}) was satellite DNA (Fig. 2c). Further purification was achieved by sucrose gradient centrifugation (Fig. 3). As expected the relative content of satellite DNA in the gradient fractions increased with the size of the chromatin, and the DNA in the most rapidly sedimenting material was over 70 % satellite DNA (Fig. 3c).

Protein composition of satellite chromatin. The protein compositions of unfractionated chromatin, satellite chromatin and satellite depleted chromatin were compared by SDS/polyacrylamide gel electrophoresis (Fig. 4a). It is apparent that there are no major differences between these fractions. There is an indication, however, that satellite chromatin is depleted in at least

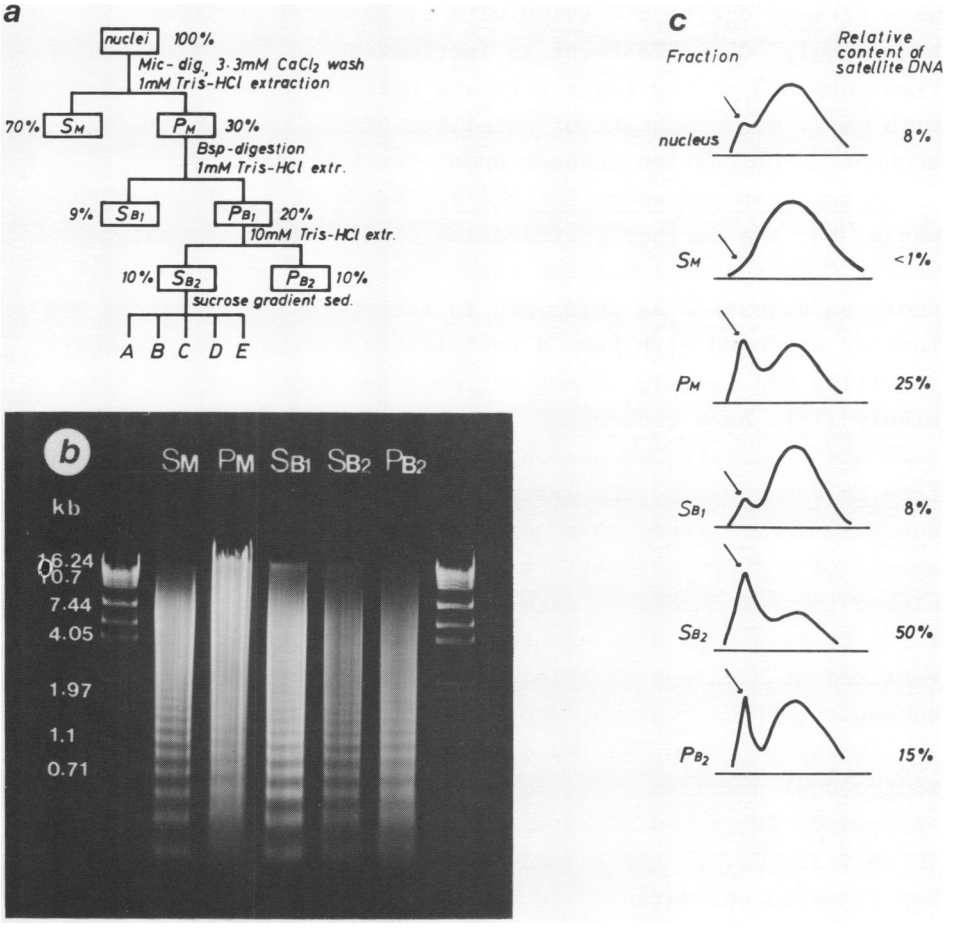

Figure 2. Fractionation of mouse satellite chromatin. a) Flow scheme of the purification of satellite chromatin, the numbers representing the relative amounts of DNA in each fraction. Digestion with micrococcal nuclease was at 1 U/ml for 20 min and with Bsp at 60 000 U/ml for 15 min. b) DNA was isolated from different fractions as indicated and analyzed in a 0.6 % agarose gel. T5 DNA digested with HindIII is used as a molecular weight reference (40). c) DNA was subjected to analytical ultracentrifugation in CsCl. In each case the arrow designates the satellite DNA while the other peak constitutes main band DNA.

HMG 17 which migrates just behind H3 in this gel system (arrow Fig. 4a). In order to substantiate this indication, proteins were extracted from satellite chromatin with 0.35 M NaCl. These are conditions at which HMG proteins are preferentially extrac-

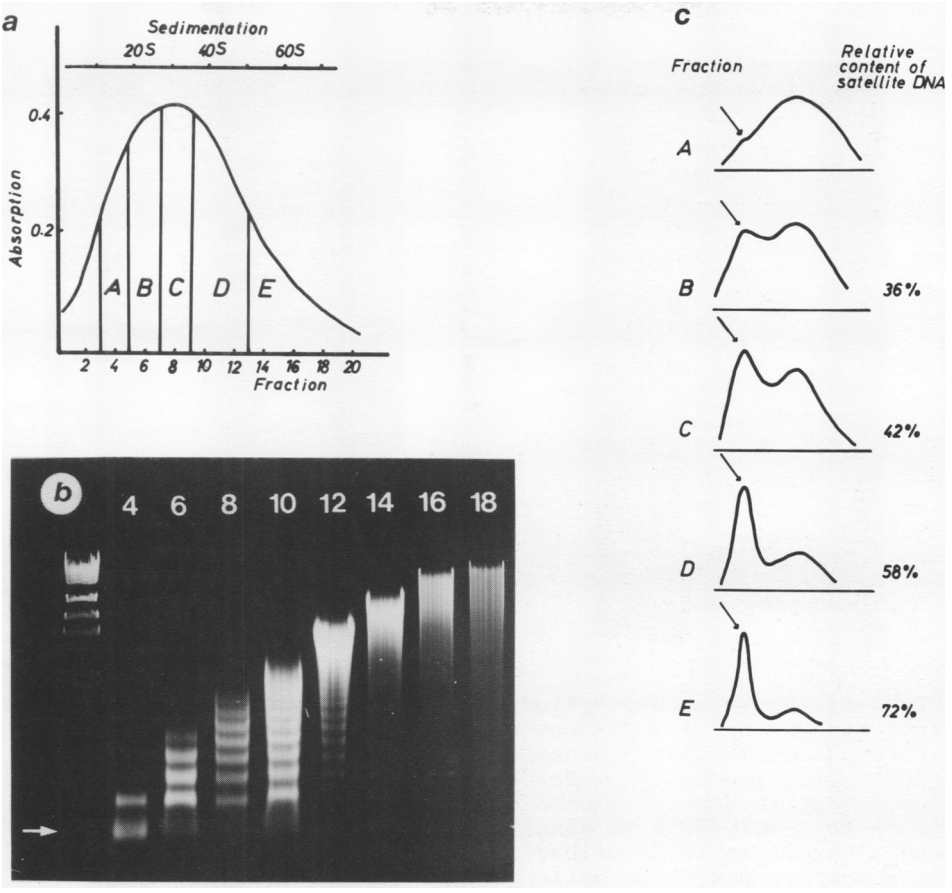

Figure 3. Purification of satellite chromatin by sucrose gradient centrifugation. a) Fraction S_{B_2} (see Fig. 2a) was applied to an isokinetic sucrose gradient (particle density 1.51, C_{mix} = 10 %) (21) containing 10 mM Tris-HCl, pH 8.0. Centrifugation was in a Beckman SW41 rotor for 15 h at 20 000 rev./min. Absorption at 260 nm is shown. b) DNA was isolated from different gradient fractions as indicated in a) and analyzed in a 0.6 % agarose gel. T5 DNA digested with HindIII as a molecular weight reference (see Fig. 2b) is shown on the left. c) DNA from regions A-E as indicated in a) was subjected to analytical ultracentrifugation in CsCl. The arrows designate the satellite DNA peaks. About 20 % of the total satellite chromatin are recovered in fraction E. The arrow in b) denotes DNA derived from mononucleosomes.

ted from chromatin (14) (see Fig. 4b, track C). The results show that HMG proteins are either absent or present in very low amounts in satellite chromatin.

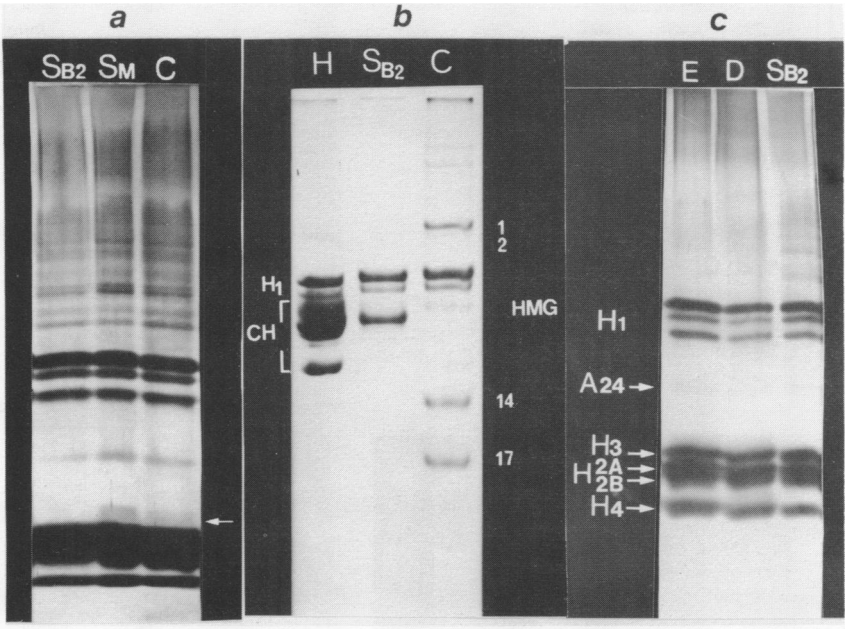

Figure 4. Protein composition of satellite chromatin. In a) and c) analysis was on 18 % SDS containing polyacrylamide gels. a) The proteins of satellite chromatin fraction S_{B2} and satellite depleted fraction S_M (Fig. 2a) are shown. Proteins are shown for comparison in track C from a chromatin fraction solubilized in 10 mM Tris-HCl, pH 8.0, after digestion of nuclei with micrococcal nuclease as described in Fig. 2. 90 % of the chromatin is solubilized with no enrichment or depletion of satellite DNA. The arrow denotes protein HMG 17 (see text). b) 0.35 M NaCl extracts from satellite chromatin fraction S_{B2} and from total chromatin (C) were analyzed in an acetic acid/urea containing polyacrylamide gel (see Materials and Methods). Purified histones are shown for comparison in track H. The positions of histone H1, core histones (CH), and HMG 1, 2, 14, and 17 are marked. c) Protein analysis of satellite chromatin fractions before (S_{B2}) and after (D,E) sucrose gradient fractionation (see Figs. 2a and 3a).

Analysis of the protein composition of gradient purified satellite chromatin shows that only trace amounts of nonhistone proteins sediment with the chromatin (Fig. 4c). It is also apparent that satellite chromatin contains all histones in about the same proportions as does bulk chromatin. There is a minor band migrating between H1 and H3 which we tentatively identify as protein A24 on the basis of its electrophoretic mobility

(22). Also this protein is present in the same amount in satellite chromatin as in bulk chromatin.

In order to analyze histone variants and modified histones, which are not resolved in SDS containing gels, we turned to acetic acid/urea gels. In Fig. 5 it is shown that the histone H1 class can be separated into five bands. They correspond to variants of histone H1 and possibly to phosphorylated H1 (16). From a comparison of Fig. 5a and b it is apparent that satellite chromatin does not differ from the other chromatin with respect to the H1 subfractions.

Variants of the core histones were analyzed in Triton-X 100 containing acetic acid/urea gels according to Zweidler (17) as shown in Fig. 6. Histone H2A, H2B and H3 variants were present in the same proportion in satellite chromatin (Fig. 6b) as in a fraction depleted in satellite DNA (Fig. 6a), and so were two bands in the H4 range which probably correspond to monoacetylated and nonacetylated histone H4. Taken together no differences in the histone composition could be detected between satellite chromatin and the rest of the chromatin.

DISCUSSION

The procedure used here for the isolation of mouse satellite DNA containing chromatin combines two different approaches. It is based on differences in conformation on one hand and differences in size on the other hand between satellite chromatin and

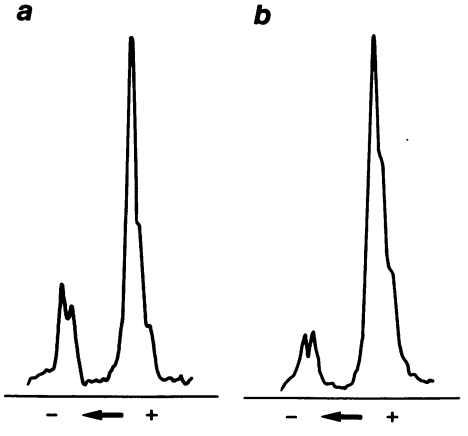

Figure 5. Analysis of histone H1 in chromatin fractions. Satellite chromatin fraction S_{B_2} (b) is compared to fraction S_M (a) (see Fig. 2a). Histones were analyzed in acetic acid/urea containing gels as described in Materials and Methods. Densitograms of the H1 regions of the gel are shown.

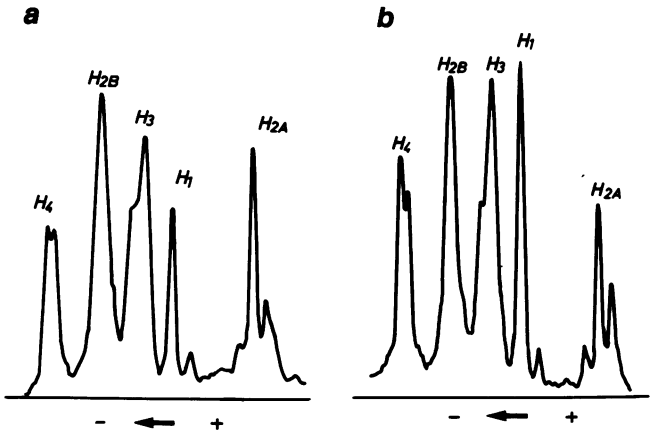

Figure 6. Analysis of histone variants in satellite chromatin. Satellite chromatin S_{B_2} (b) is compared to fraction S_M (a) (see Fig. 2a). Histones were analyzed in acetic acid/urea/Triton-X 100 containing gels as described in Materials and Methods. Densitograms are shown.

bulk chromatin after nuclease digestion.

The high compaction of satellite chromatin under our experimental conditions is strictly dependent on the prior washing of the nuclei with Ca^{++} ions. Divalent ions are known to bind tightly to chromatin and to have a powerful condensing effect. After digestion with micrococcal nuclease, Ca^{++} treated chromatin could not be solubilized in buffers of very low ionic strength even after repeated extractions. Only upon the addition of monovalent ions to the extraction buffer in concentrations between 0.5 and 10 mM did the chromatin become increasingly soluble. A possible explanation might be that the monovalent ions displace bound Ca^{++} from the chromatin. Importantly, bulk chromatin is solubilized at lower salt concentrations than satellite chromatin. Thus the latter can be enriched in the insoluble fraction. It is reasonable to assume that satellite chromatin binds Ca^{++} more strongly than bulk chromatin does, and therefore relatively higher concentrations of monovalent ions are required to solubilize the satellite chromatin. The molecular basis for such a stronger Ca^{++} binding is not clear. It might be special Ca^{++} binding proteins, alternatively elements of the superstructure of the satellite chromatin might be

responsible for the Ca^{++} binding. The fact that the differences in solubility between satellite chromatin and bulk chromatin disappear upon exposure of the nuclei to very low ionic strength prior to Ca^{++} treatment is compatible with either interpretation. Putative Ca^{++} binding proteins might be lost or redistributed, on the other hand the higher order structure of chromatin is strongly affected by the ionic environment.

Most satellite DNAs are either completely resistant to specific restriction nucleases or give regular patterns consisting of small molecular weight fragments, quite different from the continuous fragment distribution found with non-repetitive eukaryotic DNA. This difference can be exploited for the preparation of heterochromatin with restriction nucleases and subsequent extraction of chromatin (9,23-27). In the cases where satellite DNA is frequently cut the corresponding chromatin fraction is preferentially released into the soluble fraction. Further purification is achieved by sucrose gradient centrifugation or chromatography (e.g. 9,24,25). In the cases where satellite DNA is rarely cut by a restriction nuclease the satellite chromatin is retained in the insoluble fraction after digestion of the nuclei with the restriction nuclease. Since a substantial fraction of the non-satellite chromatin also remains insoluble there is usually only a moderate enrichment of satellite chromatin in this fraction (e.g. 26,27). Additional fractionation according to size presents a problem with insoluble material and therefore solubilization steps have been introduced. Mazrimas et al. have sheared the insoluble fraction through a needle in a 3 M urea containing buffer when they purified satellite chromatin from mouse brain (27). This step is undesirable if for example analyses of nonhistone proteins are intended. We have therefore not used nuclei as a starting material for digestion with restriction nuclease. Instead we first digest nuclei with micrococcal nuclease and enrich satellite chromatin in an insoluble fraction. After digestion of this material with Bsp, satellite chromatin can be solubilized simply by raising the ionic strength to 10 mM. The size difference between satellite chromatin and bulk chromatin can then be exploited by sucrose gradient centrifugation. A satellite chro-

matin fraction of high molecular weight can be produced this way which is over 70 % pure.

The histone composition of satellite chromatin is very much like that of bulk chromatin. No significant differences were detected even when histone variants present in only small amounts were compared. Billings et al. (16) have suggested that in Drosophila virilis, the phosphorylation of histone H1 might be essential to the compaction of satellite DNA into heterochromatin. In our case, phosphorylation of histone H1 is unlikely to play a role even though to prove this point rigorously one would have to rule out the possibility of histone H1 dephosphorylation during sample preparation.

Our preparation of satellite chromatin has a very low content of nonhistone proteins. This is especially obvious in the analysis of gradient purified fractions. It must be borne in mind, however, that soluble chromatin tends to be generally depleted in nonhistone proteins (28). The only significant difference detected between satellite chromatin and bulk chromatin concerns the HMG proteins which could not be detected in mouse satellite chromatin. A depletion in HMG proteins has also been reported for rat satellite chromatin prepared as a low molecular weight fraction after restriction nuclease digestion of rat liver nuclei (29), while a human repetitive DNA fraction was found to hybridize to DNA from HMG containing mononucleosomes derived from HeLa cells (33). The relative proportion of HMG proteins on the corresponding chromatin fraction is difficult to judge, however, from the hybridization signal. Our results are in contrast to those of Musich et al. who have described that in a satellite chromatin fraction from African Green Monkey nuclei histone H1 is replaced by a set of nonhistone proteins (25). Protein redistribution or degradation has not been excluded in that study, however, and might therefore be at least in part responsible for the different protein composition of the satellite chromatin preparation.

The purification scheme used here for the preparation of satellite chromatin offers a number of advantages over procedures otherwise used, especially as far as analysis of the protein composition of satellite chromatin is concerned. Harsh

conditions like high ionic strength or the presence of urea (26,27) which are known to dissociate proteins from chromatin are avoided. Satellite chromatin is isolated as a high molecular weight fraction and is not degraded to short oligonucleosome chains as in other isolation procedures involving digestion with restriction nucleases (9,23-25). The absence of characteristic differences between bulk chromatin and satellite chromatin as isolated here can therefore not be ascribed to trivial reasons such as too extensive nuclease digestion or adverse solubilization media. Nevertheless it is conceivable that key proteins are lost once chromatin is extracted from the nucleus regardless of the conditions used. It is also important to keep in mind that nonhistone proteins present in small amounts would not be detected in a screening of the type presented here. Still another possibility is raised by results obtained with Drosophila melanogaster (30) which have suggested the existence of satellite DNA associated proteins which bind so tightly that they would not be released under our experimental conditions.

The question as to what makes mouse satellite chromatin heterochromatic remains an open one. If the absence of characteristic features in the protein composition as found in our analysis is indicative of the state of heterochromatin within the cell nucleus it might be other mechanisms that are responsible for the characteristics of the heterochromatin. One interesting possibility would be that it is the specific positioning of the histones on the satellite DNA, usually referred to as "phasing" (31) which facilitates the dense packing of the heterochromatin within the nucleus.

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