A partial characterization of DNA fragments protected from nuclease degradation in histone depleted metaphase chromosomes of the Chinese hamster

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ABSTRACT

A small proportion (0.1-0.5%) of the total DNA content of native Chinese hamster metaphase chromosomes is protected from nucleolytic degradation following the removal of histones by extraction with either 0.2 N HCl or 2 M NaCl, and remains attached to the nonhistone protein core. Acid extraction followed by DNase I digestion leads to small fragments of 10-30 bases. Salt extraction followed by micrococcal nuclease digestion gives approx. 140 b.p. fragments which are undistinguishable in size from nucleosome core DNA fragments. Furthermore, DNase I treatment of salt extracted chromosomes gives DNA fragments containing single strands which are multiples of 10 bases in length, again characteristic of the nucleosome structure. Reassociation kinetics using the ^{32}P-labelled 140 b.p. fragments as probes suggests they are enriched for rapidly reassociating sequences.

INTRODUCTION

Methods have been developed for the selective removal of histones from isolated metaphase chromosomes, either by extraction with dilute hydrochloric acid (2), competition with polyvalent anions (3) or 2 M sodium chloride (4), in each case without the basic metaphase structure being lost. In the case of dilute hydrochloric acid extraction we have previously shown (2) that the nonhistone proteins remaining form a 'core' which is retained after solubilization of the bulk of the DNA by treatment with DNase I. We postulated that the small amount of DNA remaining after this treatment (0.1-0.3%) may include sequences which are recognised by certain of the residual nonhistone core proteins, and may be responsible for the establishment and/or maintenance of the metaphase state (2).

The morphological product of removing histones from metaphase chromosomes with polyvalent anions or 2 M sodium chloride is somewhat different from that obtained by acid extraction, the DNA in the former case being much less tightly packed, with relaxed loops of DNA which may be visualized in the electron microscope (4). However, the retention of a

nonhistone protein core structure is similar, and preliminary analysis of the residual proteins by SDS gel electrophoresis suggests that they are the same in the two instances (5). The object of this communication is to present some results of attempts to characterize and compare the residual DNA found in metaphase chromosomes depleted of histones both by treatment with dilute acid and extraction with 2 M sodium chloride, and subsequently digested with nucleases to remove the bulk of the DNA.

MATERIALS AND METHODS

A. Isolation of Chromosomes, Histone Extraction by 0.2 N HCl and Nucleolysis.

Metaphase chromosomes, either nonradioactive or containing DNA labelled *in vivo* with ^{3}H-methyl thymidine (Radiochemical Centre, Amersham) were isolated from a clone (K2) derived from the Don Chinese hamster cell line as described previously (2). Histones were extracted at 0°C for 4 h with 0.2 N HCl, also as described (*ibid*), and the acid treated chromosomes were finally suspended in 10 mM Tris-HCl, pH 8, containing 1 mM $MgCl_2$, 1% 2-methylpentan-2, 4-diol ('hexylene glycol') and 0.1% Triton X-100 ('Tris-Mg medium') at a concentration of approx. 200 µg DNA/ml (4 $\bar{A}_{260}$). Digestion by DNase I (Worthington Biochemical Corp.) was performed by the addition of 25 µg/ml enzyme and incubating in ice for 1 h, after which the reaction was stopped by the addition of EDTA-Na, pH 7, to 10 mM, and the DNase I chromosome 'cores' sedimented by centrifugation at 3400 rpm. The supernatant was decanted and the pellet washed twice by resuspending in 10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% hexylene glycol, 0.1% Triton X-100 ('Tris-EDTA medium') and centrifuging, before finally resuspending in 1 ml of Tris-EDTA medium prior to phenol extraction (see below). Digestion with micrococcal nuclease (Worthington Biochemical Corp.) was carried out for 1 h in ice at a concentration of 600 u/ml enzyme in Tris-Mg medium with the addition of 1 mM $CaCl_2$. The micrococcal nuclease 'cores' were then treated exactly as in the case of DNase I cores described above.

B. Histone Extraction by 2 M Sodium Chloride and Nucleolysis.

The procedure used for salt extraction of histones was a modification of that described by Paulson and Laemmli (4). To 2.7 ml of chromosome suspension in 10 mM Tris-HCl, pH 8, 2 mM $CaCl_2$, 1% hexylene glycol, 0.1% Triton X-100 ('Tris-Ca medium') at 4°C, containing approx. 200 µg of DNA, is added 0.3 ml of 0.1 M EDTA-Na, pH 7. 2 ml of cold 5 M NaCl is then added, and mixed in by gently inverting the tube several times (the suspension

becomes quite viscous). The suspension is then layered gently with a wide bored Pasteur pipette into a 1.5 cm x 9.5 cm centrifuge tube on the surface of 8 ml of Tris-EDTA medium {see (A)} containing 2 M NaCl and 2.5% (w/v) sucrose, which itself floats on a cushion of 1 ml of Tris-EDTA medium containing 2 M NaCl and 0.6 M metrizamide (Nyegaard and Co., Oslo). After standing for 30 min. at 4°C the tube is centrifuged for 4 h at 2000 rpm in an MSE 6-place 15 ml swinging bucket rotor at 4°C, at the end of which the dehistonized chromosomes collect as a band at the metrizamide/sucrose boundary. The band can be collected by inserting a large bore hypodermic needle below the band and removing into a syringe (a small band of viscous material often collects at the top of the 2.5% sucrose and is discarded).

The dehistonized chromosomes were then desalted by dialysis for 16 h at 4°C against Tris-Mg medium after which they were digested with either DNase I or micrococcal nuclease under similar conditions to those described above under (A). After enzymic digestion and addition of EDTA, the chromosome cores were separated from the bulk of the DNA as follows. The sample (approx. 2 ml) is layered into a 1.5 cm x 9.5 cm centrifuge tube on the surface of 10 ml of Tris-EDTA medium containing 2.5% sucrose, supported on a cushion of 1 ml of Tris-EDTA medium containing 0.9 M metrizamide. The cores are concentrated at the metrizamide/sucrose boundary by centrifugation for 3 h at 3000 rpm in a 6-place MSE 15 ml swinging bucket rotor at 4°C. The cores are collected as described above, ready for phenol extraction.

C. Isolation of DNA from Chromosome Cores.

The DNA remaining in chromosomes dehistonized and nuclease treated in any of the ways described above was isolated by phenol extraction in the presence of sodium dodecyl sulphate (SDS). The chromosome cores suspended in Tris-EDTA medium were made 0.5% (w/v) in SDS and warmed at 37°C for 10 min. The denatured cores were then extracted three times with an equal volume of water saturated redistilled phenol, the aqueous phase then being ether extracted twice to remove excess phenol. The nucleic acids were precipitated by the addition of one tenth volume of 5 M NaCl followed by 2½ volumes of absolute ethanol and left at -20°C overnight. The precipitate was collected by centrifugation at 30,000 rpm for 30 min. The supernatant was decanted and the pellet dried *in vacuo* before being dissolved in a small volume (5-10 µl) of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The DNA was then freed of RNA in one of two ways. Either sodium hydroxide was added to a final concentration of 0.2 N and incubation carried out at

37°C for 16 h in a sealed glass capillary, after which the alkali was neutralized with an appropriate volume of N HCl, and the sample diluted with 1 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and precipitated by ethanol as before; or alternatively, RNase A (Worthington Biochemical Corp.) and RNase T_2 (Sankyo Co Ltd, Japan) were added to final concentrations each of 0.1 mg/ml, and the sample incubated for 16 h at 37°C in a sealed capillary. The sample was then phenol extracted once to remove the enzymes, diluted, and ethanol precipitated as before.

D. ^{32}P 5'-end labelling with T4 polynucleotide kinase.

DNA fragments were labelled at their 5'-positions by ^{32}P-phosphate transferred from high specific activity γ-^{32}P-ATP (Radiochemical Centre, Amersham; 3000-4000 Ci/mmole) by the action of T4 polynucleotide kinase (P-L Biochemicals Inc.) by a modification of the method of Richardson (6). DNA cleavage products of micrococcal nuclease already have a free 5'-end, but DNase I products first require the removal of their 5'-phosphate. The DNA, freed of RNA as described above, was dissolved in 10 µl of 10 mM Tris-HCl, pH 8.9, to which was added 1 µl of a 5 mg/ml solution of bacterial alkaline phosphatase (Sigma Chemical Corp.), and phosphatase action allowed to proceed at 37°C for 30 min. The phosphatase was then inactivated and removed by extraction with an equal volume of water saturated phenol, and excess phenol removed by ether extraction.

For ^{32}P-labelling, the DNA sample, dissolved in 10 µl of water, or Tris buffer after phosphatase treatment, was mixed with 10 µl of 0.1 M Tris-HCl, pH 7.5, 0.02 M $MgCl_2$, 0.01 M dithiothreitol, 50% glycerol, containing 50 µCi of γ-^{32}P-ATP. 1 u of polynucleotide kinase was added, and incubation carried out at 37°C for 30 min. The labelled DNA was then separated from the excess γ-^{32}P-ATP by gel filtration through a 0.25 cm x 25 cm column of Sephadex G100 (Pharmacia) equilibrated in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The fast running radioactive peak containing the labelled DNA was collected and the DNA precipitated by ethanol and sedimented by centrifugation as described above in (C).

E. Gel Electrophoresis

Polyacrylamide gel electrophoresis of ^{32}P-labelled DNA fragments was performed in 0.15 cm x 20 cm x 20 cm slab gels. When it was wished to run double stranded DNA fragments, gels containing 20% acrylamide and 0.067% NN'-methylene-bisacrylamide ('bisacrylamide') were used, polymerized with ammonium persulphate and N,N,N',N'-tetramethylethylene diamine, and run in

40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 8.3, electrophoresis buffer, at room temperature. Ethanol precipitated samples were dried in vacuo and loaded onto the gel in 5-10 µl of 1/5 concentration electrophoresis buffer containing 10% sucrose and a small quantity of bromphenol blue marker dye, and electrophoresed at 40 mA until the blue marker had migrated approx. two-thirds the length of the gel. The radioactivity was then located by autoradiography.

If it was required to run DNA under denaturing conditions, a gel containing 15% acrylamide, 0.5% bisacrylamide and 7 M urea was utilized, using as electrophoresis buffer 0.09 M Tris-borate, 2 mM EDTA, pH 8.3. Samples were dissolved in 5 mM EDTA-Na, pH 7, in 90% formamide, containing bromphenol blue and xylene cyanol blue marker dyes, heated to 100°C for 5 min., then loaded onto the gel, and run at 16 mA at room temperature until the bromphenol blue had migrated approx. two-thirds the length of the gel. Radioactive bands were located as before by autoradiography.

F. Pyrimidine Tract 'Fingerprinting'.

Depurination of DNA fragment samples followed by ether extraction to remove diphenylamine was performed according to Ling (7). The aqueous solution of pyrimidine tracts was then lyophilised, and the dried DNA phosphatased and ^{32}P end labelled as described in (D) above. The ^{32}P-oligonucleotides were then separated by the two-dimensional electrophoresis/chromatography system described by Ling (7).

G. Restriction Analysis.

The ^{32}P end labelled 140 b.p. fragment preparations obtained from micrococcal nuclease treatment of salt dehistonized chromosomes (see Results) were tested for the presence of restriction sites by diluting with nonradioactive carrier E. coli DNA to give a suitable DNA concentration, and incubating under appropriate conditions with a series of restriction enzymes obtained from Biolabs Inc. The DNA was then rerun on nondenaturing 20% polyacrylamide gels and radioactivity located by autoradiography as in (E).

H. Reassociation Kinetics Analysis.

For use as a probe in reassociation experiments, double-stranded 140 b.p. DNA fragment preparations were internally labelled with ^{32}P using the 'nick-translation' technique. After labelling with DNA polymerase I and inactivation of the enzyme as described previously (8) the deoxyribonucleoside triphosphates were removed by Sephadex G100 filtration

as above (D). The ^{32}P-labelled DNA fragments were then separated from the small amount of high molecular weight (presumably branched chain) product always obtained with nick-translation, by electrophoresis on a nondenaturing 20% polyacrylamide gel as described in (E). The radioactive 140 b.p. fragment band was then cut from the gel and eluted electrophoretically (9).

The reassociations were driven by Chinese hamster DNA (CH DNA) isolated from Don K2 cells by the procedure of Walker and McLaren (10). The reaction mixture also contained a quantity of in vivo ^{3}H-labelled CH DNA to monitor the rate of reassociation of the driving DNA, and the total concentration of DNA was kept constant at different CH DNA concentrations by the addition of unlabelled E. coli DNA (Sigma Chemical Corp.). All DNA was sheared to approx. 500-800 b.p. by sonication before use. Reassociation reactions were carried out in 200 µl Micropet disposable glass pipettes (Clay Adams). The tubes were sealed in a Bunsen flame at one end, and in a final volume of 94 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, were introduced 2-4 x 10^{3} dpm of the ^{32}P-labelled 140 b.p. fragment preparation under study, an approx. equal number of dpm of ^{3}H-labelled CH DNA, a quantity of nonradioactive CH DNA to give an aggregate CH DNA concentration in the range 0.05 - 30 $\bar{A}_{260}$, and nonradioactive E.coli DNA to give a total DNA concentration of 30 $\bar{A}_{260}$, if necessary. (The absorbance at 260 µm of the ^{32}P-labelled 140 b.p. fragment preparations were below the limit which we could measure, and their contribution to the DNA concentration was ignored). The tubes were then sealed at the other end, and immersed in a boiling water bath for 5 min. to allow DNA strand separation to occur. The tubes were then cooled quickly in ice, the top broken off, and 6 µl of 5 M NaCl added and mixed in, to give a final salt concentration of 0.3 M. The top was resealed, and reassociation carried out at 60°C, removing 15 µl aliquots for assaying the extent of reassociation at intervals, and resealing the tube after each removal. In this way, the $C_{o}t$ range between approx. 10^{-2} to 10^{3} (11) could be covered in a few days.

The extent of reassociation of the DNA was assayed by its resistance to degradation by the single-strand specific nuclease, S1. The nuclease was prepared according to Sutton (12). Control experiments showed the enzyme preparation to be highly specific for single-stranded DNA under the conditions used, and efficient at the DNA concentration employed for the reassociation assay. 5 µl of the 15 µl aliquot removed from the reassociation tubes was added to 1 ml of cold 5% trichloroacetic acid (TCA)

for estimation of the total precipitable radioactivity. The remaining 10 µl was added to 100 µl of 0.03 M sodium acetate, 0.01 M NaCl, 0.01 mM $ZnSO_4$, pH 4.5, and incubated with a three-fold excess of S1 nuclease (based on control assays) for 1 h at 37°C, after which the S1 resistant material was precipitated with TCA as above. TCA precipitates were filtered onto Whatman GF/C filters, washing with 5% TCA followed by 95% ethanol, dried, and counted in toluene scintillator separating ^3H and ^{32}P into two non-overlapping channels in a Beckman liquid scintillation counter.

RESULTS

In order to detect the small quantity of DNA remaining in metaphase chromosome cores after removal of histones by extraction with 0.2 N HCl and subsequent liberation of the bulk of the DNA by digestion with DNase I (2), we have used 5'-end labelling by polynucleotide kinase and high specific activity γ-^{32}P-ATP (6). A disadvantage of this method is that any contaminating RNA is also labelled with high efficiency, and must therefore be removed before kinasing is attempted. Metaphase chromosomes as prepared here contain RNA, the amount varying from preparation to preparation (2). Extensive washing with standard chromosome media does not release the RNA, and the procedures for removing histones and the bulk of the DNA do not completely remove this RNA either. Whether or not the RNA may be an integral part of the chromosome it is not proposed to discuss here, except insofar as its presence affects our methods for labelling the residual DNA. Incubation of the total nucleic acids extracted from the cores with RNase A under standard conditions fails to eliminate it completely, suggesting that it possesses base-paired secondary structure. In this respect the chromosomal RNA resembles tRNA, and indeed the partial RNase A digestion patterns for the two are quite similar (unpublished observation) indicating perhaps that the chromosomal RNA is at least in part adsorbed tRNA species. The most reliable way of eliminating RNA from the nucleic acid fraction is to incubate for 16 h in 0.2 N NaOH at 37°C, but this has the disadvantage of denaturing d.s.DNA. To avoid denaturing the DNA we have alternatively used a combined overnight digestion with RNases A and T_2 at 37°C, after which we have been unable to detect any residual RNA.

When the residual DNA from acid extracted Chinese hamster metaphase chromosomes digested with DNase I is isolated, freed of RNA, ^{32}P end labelled and electrophoresed through a 20% nondenaturing polyacrylamide gel, the DNA fragments migrate as in slot (a) of the autoradiograph depicted in Figure 1.

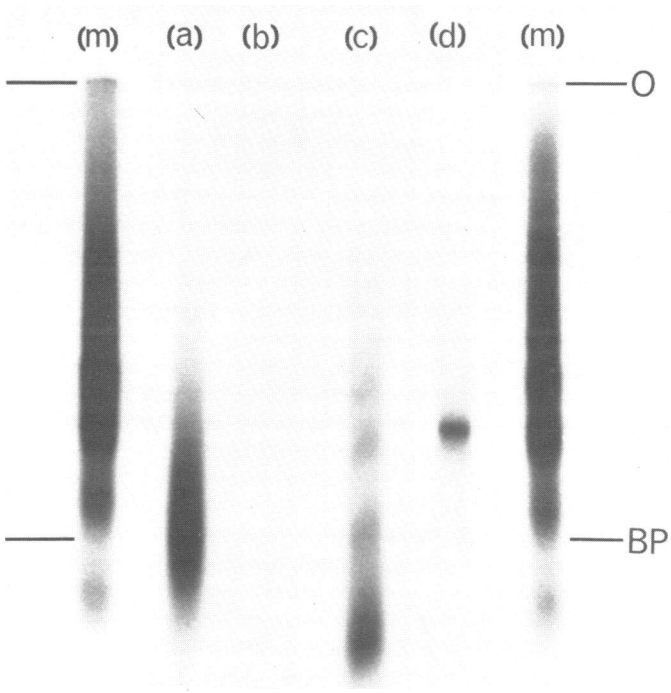

Figure 1

Autoradiograph of DNA protected from nuclease degradation in dehistonized Chinese hamster metaphase chromosomes, ^{32}P end labelled and electrophoresed on a 20% polyacrylamide nondenaturing gel as described in Materials and Methods. (a) Chromosomes dehistonized by 0.2 N HCl, treated with DNase I; (b) dehistonized by 0.2 N HCl, treated with micrococcal nuclease; (c) dehistonized by 2 M NaCl, treated with DNase I; (d) dehistonized by 2 M NaCl, treated with micrococcal nuclease. Channels (m) contain samples of ^{32}P end labelled DNA from a DNase I digest of interphase chromatin, used as a calibration marker. O indicates the origin of migration and BP indicates the position of the bromphenol blue marker dye.

The same sample run under denaturing conditions on a 15% polyacrylamide-urea gel is shown in slot (a) of Figure 2. The marker run in slots (m) of both Figs. is the DNA extracted from interphase chromatin which has been digested with DNase I, showing the typical pattern of single-stranded DNA fragments, each an integral multiple of 10 bases (13). Thus the protected DNA from the chromosome cores is shown to have a distribution of fragment sizes from approximately 10 up to 30 bases, with a peak around 20 bases. Under similar digestion conditions DNase I degrades purified DNA to fragments of less than 10 bases (not shown here).

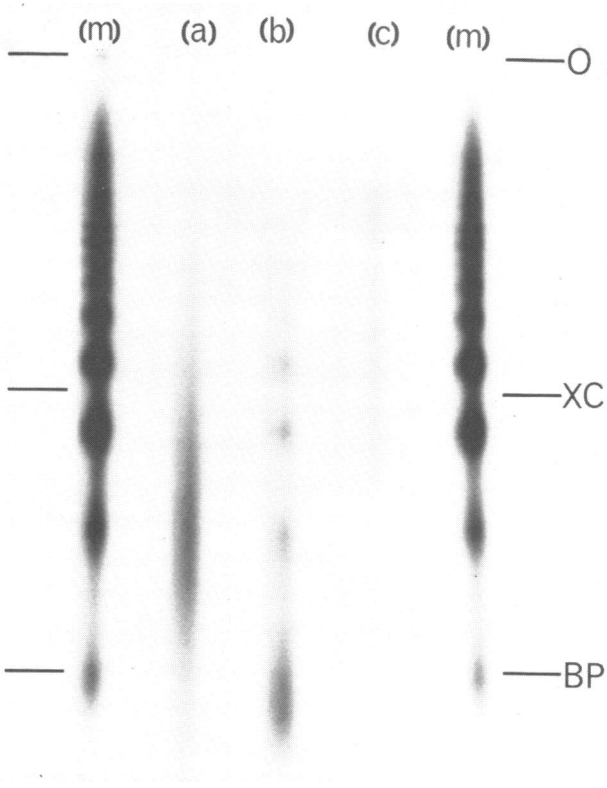

Figure 2

Autoradiograph of some of the DNA samples of Fig. 1, denatured and electrophoresed on a 15% polyacrylamide-7 M urea gel. (a), (b), (c) and (m) are described under (a), (c), (d) and (m) respectively in the legend to Fig. 1. O indicates the origin, and XC and BP indicate the positions of the xylene cyanol and bromphenol blue marker dyes respectively.

We attempted to determine if the protected DNA fragments we had isolated were enriched for particular sequences (see Discussion) by performing depurination analysis as described by Ling (7). The resulting pyrimidine tract 'fingerprint' is shown in Figure 3. The pattern of spots is indistinguishable from that which would be obtained from a random set of DNA sequences.

If HCl extracted metaphase chromosomes are incubated at $0^{o}C$ in the presence of micrococcal nuclease, core structures are obtained which are morphologically indistinguishable from those produced by DNase I. The DNA extracted from cores produced in this way, treated exactly as for DNase I

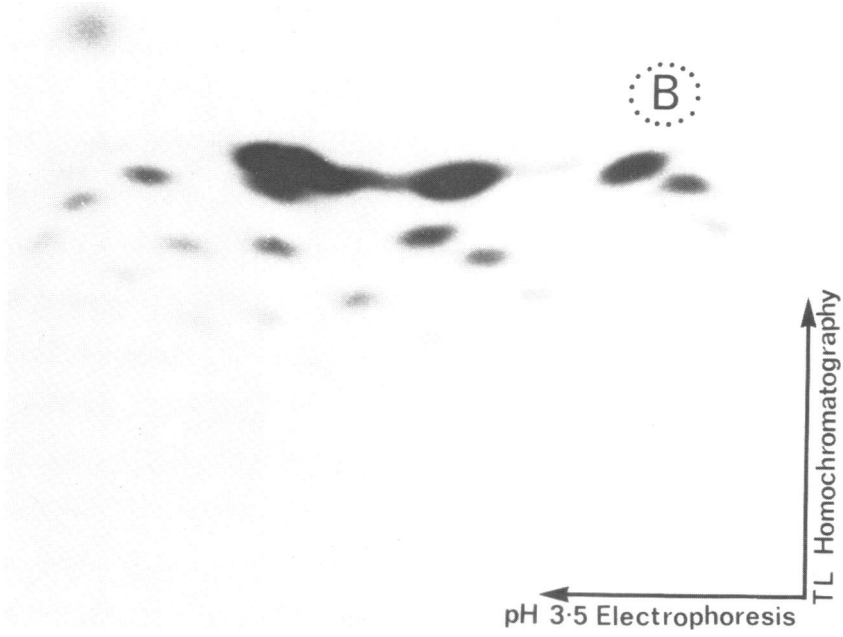

Figure 3

Autoradiograph of a two dimensional separation of the ^{32}P end labelled depurination products (7) of DNA extracted from Chinese hamster metaphase chromosomes dehistonized by treatment with 0.2 N HCl and digested by DNase I.

cores, is run in slot (b) of Fig. 1. This result is typical, and shows that very little DNA is protected from micrococcal nuclease degradation.

Because of the difficulty in characterizing the very small DNA fragments protected from DNase I degradation of acid extracted metaphase chromosomes (e.g. reassociation or hybridisation techniques are not possible), an alternative method of obtaining larger protected DNA fragments in dehistonized metaphase chromosomes was sought. We had observed that if histones were removed from native metaphase chromosomes by treatment with 2 M sodium chloride by the method of Paulson and Laemmli (4), and the resulting structures treated with DNase I or micrococcal nuclease, core structures are obtained similar to those following acid extraction and nucleolysis. In the case of chromosomes depleted of histones by extraction with 0.2 N hydrochloric acid, we were previously able to make a reasonable estimate of the quantity of DNA remaining with the nonhistone protein cores after degradation of the bulk of the DNA by DNase I: 0.1-0.3% of the DNA content

of native chromosomes, depending on the ionic conditions of the nuclease treatment (2). However, after extraction of histones by 2 M sodium chloride, owing to the different properties of the resulting structures, we have only been able to obtain a rough indication of the quantity of DNA remaining after nucleolysis, as a fraction of the DNA content of native metaphase chromosomes, and this is in the region of 0.1-0.5% for both DNase I and micrococcal nuclease treatments (see Discussion).

In order to determine the size of the residual DNA after salt extraction and nucleolysis, the cores were phenol extracted and the nucleic acid fraction incubated overnight with RNases A and T_2 to remove RNA without denaturing the DNA. The DNA was then labelled by kinasing as before. Slots (c) and (d) of Fig. 1 and (b) and (c) of Fig. 2 show the results for DNase I and micrococcal nuclease run on non denaturing and denaturing gels respectively. It can be seen that DNase I produces a 10-base interval 'ladder' of single-stranded DNA fragments similar to that obtained by the action of DNase I on interphase chromatin (slots (m) in Figs. 1 and 2). Micrococcal nuclease gives a double-stranded DNA fragment which has been sized on gels (not shown here) at approx. 140 base pairs, and is indistinguishable in mobility from the nucleosome core DNA fragment obtained from limit digests of interphase chromatin with micrococcal nuclease (14). This is strongly suggestive that there are nucleosomes still present in chromosomes depleted of histones by the 2 M sodium chloride technique even though protein gels fail to detect significant quantities of residual histones (5). These nucleosomes are therefore present in very low yield, perhaps stabilized within the nonhistone protein core. Our method for isolating the dehistonized chromosomes should not allow reconstitution.

The size of the protected fragments from salt extracted, micrococcal nuclease digested chromosome cores is suitable for using in reassociation experiments. For this purpose the core DNA was internally radioactively labelled with ^{32}P by 'nick-translation' and mixed with <u>in vivo</u> ^{3}H-labelled total Chinese hamster DNA (used to drive the reassociation) before denaturation. The extent of reassociation at different $C_o t$ values (11) was then measured by resistance to degradation by the single-strand specific nuclease S1 (12). The result of a typical experiment is shown in Fig. 4 where the reassociation of total DNA (^{3}H counts) and protected core DNA fragments (^{32}P counts) are plotted against the total $C_o t$ value. The curve for total DNA is similar in shape to that obtained by other workers for Chinese hamster DNA using the more conventional technique of

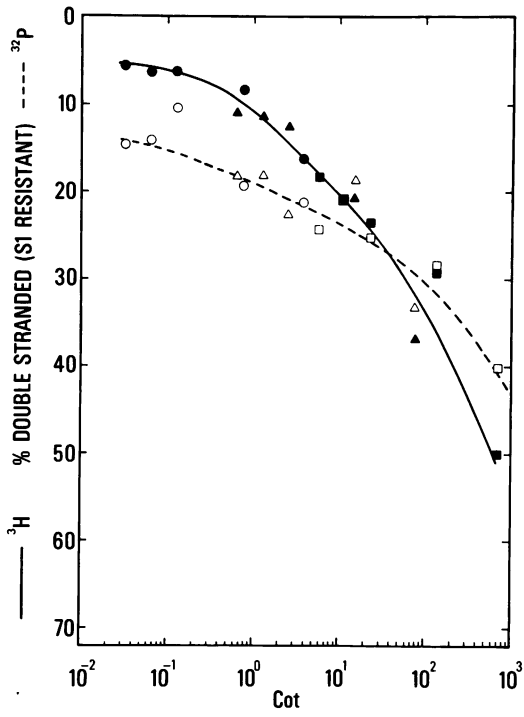

Figure 4

$C_o t$ curve of ^{3}H-labelled Chinese hamster DNA (solid line and filled symbols) including a probe of ^{32}P-labelled 140 b.p. DNA fragments from salt extracted, micrococcal nuclease digested chromosome cores (dashed line and unfilled symbols). The conditions of reassociation and assay of double strandedness with S1 nuclease are described in Materials and Methods. Three initial Chinese hamster DNA concentrations were employed in constructing this curve: 0.065 $\bar{A}_{260}$ (circles), 1.3 $\bar{A}_{260}$ (triangles) and 11.8 $\bar{A}_{260}$ (squares). The units of $C_o t$ are moles l^{-1} sec.

hydroxylapatite chromatography (15), and in our view this validates the use of the somewhat simpler S1 nuclease technique described here. Although the exact paths of the reassociation curves can only be estimated because of the scatter introduced by sampling errors, it is apparent that at the lowest $C_o t$ value measured (approx. 3×10^{-2}) there is a 2½-fold higher participation in S1 resistant DNA by the core DNA fragments when compared with the total Chinese hamster DNA curve. As reassociation proceeds the ^{32}P and ^{3}H curves appear to coincide or even, perhaps, cross over, although the number of points in this region are not sufficient for certainty. A comparison of the relative degrees of reassociation at different $C_o t$ values for three different preparations of the 140 b.p. DNA, using the total DNA curve as standard, is shown in Figure 5. It can be seen that a similar trend occurs in each case,

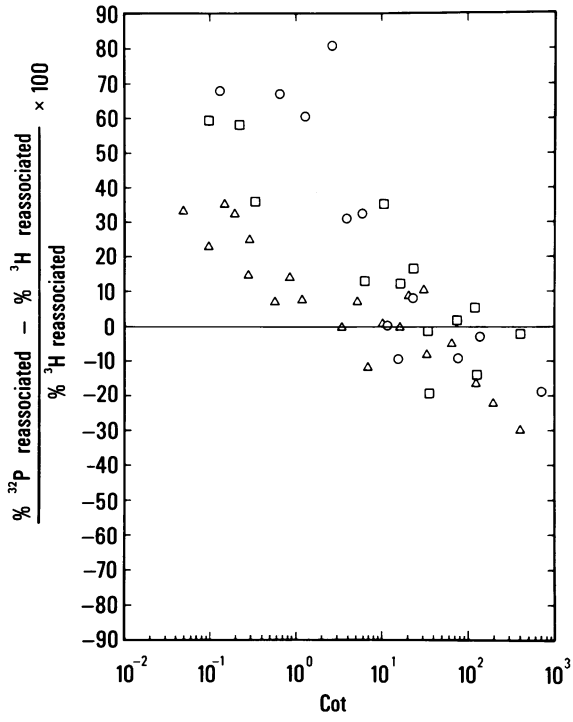

Figure 5

The enrichment for rapidly annealing sequences in the 140 b.p. DNA fragments from salt extracted, micrococcal nuclease digested chromosome cores (^{32}P), compared with total Chinese hamster DNA (^{3}H); different symbols represent independently prepared fragment preparations. The points indicated by circles were derived from the data of Fig. 4.

the only difference being in the degree of enrichment for rapidly reassociating sequences at low $C_o t$.

A depurination fingerprint of the 140 b.p. DNA was performed as before for the acid extracted core DNA, but with the same result: apparently random DNA sequences. The fragments were also incubated with a series of restriction endonucleases, but with no indication of specific cleavage occurring in any case. The restriction enzymes tested were: Alu I, Hae III, Hinf I, Hha I, Hpa II, Mbo I, Pst I, Sal I, Taq I, Xba I.

DISCUSSION

In a previous publication (2) we suggested a model to account for the specific packaging of chromatin in metaphase chromosomes, based on our own observations and those of Laemmli and co-workers (3,4), that a nonhistone protein 'core' appears to be responsible for maintaining the structure of

histone depleted metaphase chromosomes. We proposed that specific sequences might be found spaced along the genome capable of being recognised and bound by a small class of nonhistone proteins which, when assembled, form the backbone of the metaphase chromatid. This hypothesis was supported by the finding that upon DNase I treatment of chromosomes depleted of histones by extraction with dilute hydrochloric acid, a small quantity of DNA (0.1-0.3% of the original) was retained together with the nonhistone protein core. We believe that the additional findings reported here lend further support to our model for metaphase chromosome structure, although by no means proving it.

The DNA isolated from metaphase chromosome cores prepared by acid extraction of histones followed by DNase I digestion has a size distribution (10-30 bases with a peak at approx. 20 bases) typical of what one might expect for a population of DNA fragments protected from nuclease digestion by the specific binding of protein molecules (cf lac operon repressor binding site (16)). Under identical digestion conditions purified DNA is degraded to much smaller oligonucleotides which would be lost to a large extent during the ethanol precipitation and Sephadex chromatography steps to which the samples of Fig. 1 have been subjected. Some interaction, therefore, between the DNA and core proteins must be responsible for the protection, and the size distribution is small enough to suggest a specific rather than a nonspecific effect. Although a nondenaturing gel is used for the separation shown in Fig. 1(a), it is clear by comparison with Fig. 2(a) that the fragments are migrating as a single-stranded species. Double-stranded DNA composed of single strands of this size would be relatively unstable, and the manipulations involved in preparing the DNA for electrophoresis could be expected to cause strand separation (a similar result is obtained for the DNase I digest of interphase chromatin shown in Fig. 1(m), where although no denaturation step is employed, the smaller fragments are migrating as single-stranded DNA). We cannot explain why we obtain such a low yield of protected fragments when using micrococcal nuclease rather than DNase I to generate core structures from acid extracted metaphase chromosomes. The cores themselves are morphologically indistinguishable from DNase I cores, and appear to be obtained in the same yield. This suggests that DNA itself is not a necessary component of the core structure.

We believe it is significant that metaphase chromosomes treated with 2 M sodium chloride retain some DNA in structures which are indistinguishable from nucleosomes as assayed by the DNA fragment patterns obtained after digestion by micrococcal nuclease or DNase I. It has been assumed that 2 M

sodium chloride completely dissociates histones from chromatin (17,4). Other workers (18) and ourselves (5) have also failed to detect significant quantities of residual histones in 2 M salt extracted chromosomes on protein gels. Thus it would appear that the bulk of histones are removed by the salt treatment, but that certain nucleosomes are stabilized within the nonhistone protein backbone. A possible trivial explanation for the retention of some nucleosomes in 2 M sodium chloride and the fact that they contain DNA enriched for rapidly reassociating sequences would be that the nucleosomes formed from, for example, regions of highly repetitive DNA possess higher than average stability in salt. However, the authors know of no previously published data which would indicate this to be the case. We have no way of telling at present if the DNA contained in the stabilized nucleosomes is related to the protected DNA found in metaphase chromosomes depleted of histones by treatment with dilute hydrochloric acid. There are differences between chromosomes depleted of histones by the two techniques, which are described in detail elsewhere (5). As far as the DNA is concerned, the differences may be summarised as follows. As shown previously by fluorescence microscopy (2) extraction of the histones with 0.2 N HCl leaves the DNA relatively tightly packed around the central nonhistone protein core. On the other hand, extraction with 2 M sodium chloride leaves the DNA in a relaxed, highly diffuse state. This can clearly be seen in the electron micrographs of Paulson and Laemmli, where the DNA loops extend radially up to approx. 10 μm from the central core region (4). This difference in state of DNA compaction appears to be an irreversible result of the method of dehistonization: acid extracted metaphase chromosomes spread on cytochrome c films either with or without 2 M NaCl pretreatment, and prepared for electron microscopy demonstrate that the bulk of the DNA remains tightly packed around the central core, with very little extending as relaxed loops (5). However, we would like to think that the structural elements are the same in both cases, and therefore we would hope that if there are indeed base sequences on the DNA which are being recognised by nonhistone proteins, that they are present in both the DNA from the stabilized nucleosomes found in salt extracted chromosomes and the protected DNA after acid extraction.

A practical result of the different morphologies of acid extracted and salt extracted dehistonized metaphase chromosomes is that whereas acid extraction leaves the DNA intact, in structures which are stable to repeated centrifugation and resuspension in suitable media with very little DNA loss (2), the DNA of salt extracted metaphase chromosomes is extremely sensitive

to shear, and the recovery of DNA after the step gradient purification of dehistonized chromosomes is often quite low (10-50%, shown by recovery of radioactivity from chromosomes labelled in vivo with ^{3}H-methyl thymidine) particularly when attempting to prepare large quantities of dehistonized chromosomes. Although we would attribute most of this loss to shear from individual chromosomes, it is possible that some is also accounted for by complete uncoiling of a proportion of the chromosomes when subjected to the 2 M salt treatment, induced perhaps by proteolytic damage to the nonhistone core. Although systematic physical counting experiments to determine the extent of this loss are complicated by the fact that chromosomes depleted of histones by salt tend to aggregate, especially in more concentrated suspensions, we do not consider it to amount to as much as 50% in most experiments, although occasionally markedly higher losses have been observed in particularly poor chromosome preparations. The difficulty of apportioning the losses between these two routes leads to a large possible error in estimating the proportion of DNA present in native chromosomes that is retained in the core after salt extraction and nuclease degradation. However, using metaphase chromosomes containing ^{3}H-labelled DNA, and correcting the recovery of label by attempting to apportion losses incurred in either of the ways discussed above for particular chromosome preparations, we would estimate the figure to be somewhere between 0.1-0.5% for both DNase I and micrococcal nuclease treatments.

Apart from the indication in the reassociation data shown in Figs. 4 and 5, that the protected nucleosome DNA from salt extracted chromosomes is enriched for rapidly reannealing sequences, other attempts so far to show that the DNA protected from nucleolytic degradation both in salt and acid extracted chromosomes contain specific sequences have proved unsuccessful. While a more positive result would have been encouraging as further evidence in favour of our model of metaphase chromosome structure, using the characterization techniques we have employed, it would probably have been entirely fortuitous if we had detected any departure from randomness. If we consider the model in more detail, we would propose that each structural element of the metaphase chromosome, i.e. each loop as visualised under the electron microscope (4) should contain at least one recognisable sequence to attach to the nonhistone protein backbone. If the simplest possibility is considered, in which there is a single sequence repeated once in each loop, then a rough estimate of the length of the putative sequence can be made. According to Paulson and Laemmli (4), the weight average circumference of a

single loop as visualised by the electron microscope is approx. 23 μm i.e. 70,000 base pairs of DNA. Hence the size of the sequence would have to be such that on average it would be unlikely to occur by chance alone more frequently than once in 70,000 bases. If one assumes an equal probability for each of the four bases as a first approximation, then an n-long sequence would have a probability of $1/4^n$, which reduces to less than 1/70,000 at n=9. We would therefore suggest that this is approximately the minimum sequence length which would satisfy our model, and that although the putative protein recognition sequence could be longer it need not necessarily be so. Such a sequence length is not unreasonable as a protein recognition signal (see, for example, ref 16). If we take as a likely size approx. 10 base pairs, then even in the short fragments obtained by protection from DNase I degradation in acid extracted chromosomes, the mean fragment size of 20 bases would only be 50% enriched for the putative sequence, the remaining 50% being adjacent sequences selected at random from the total genome. Thus unless the recognition site had a particularly long pyrimidine tract, it would be extremely difficult to detect in the depurination fingerprint. In the case of the 140 base pair protected DNA fragments, detection would be virtually impossible. Finding a restriction site within a 10-long sequence is also highly improbable. Even reassociation kinetics would be unlikely to give a clear answer, as any reassociation of a 10 base sequence would be unstable and the bulk of the fragments would reassociate according to their random sequence complement. Such instability may account for the variability in the quantity of rapidly reassociating sequences that we find from experiment to experiment, perhaps through small differences in conditions of reassociation or S1 nuclease digestion of which we are unaware. The possibility that the putative sequence might not be consecutive could further impede the analysis. Another possibility is that, in common with many other protein recognition sequences, the putative specific sequences contained in the 140 base pair fragments might possess dyad symmetry (inverted repetition), and the very early reassociation that we observe may be partly or wholly accounted for by snap-back structures. If this is the case then the variability in amount could be caused by single strand nicks leading to separation of the two halves of the sequence occurring to different extents in different 140 base pair fragment preparations. The sample run on the denaturing gel of Fig. 2(c) shows that there is some nicking in this particular preparation.

At present, the exact nature of the sequences involved can only be

surmised, but it is clear that the 140 base pair fragments protected from micrococcal nuclease degradation are enriched for rapidly reassociating DNA compared with total Chinese hamster DNA. Razin et al (18) have suggested that the DNA adjacent to the attachment sites in salt extracted mouse metaphase chromosomes is enriched for satellite and other repeated sequences. Their data, however, is based on highly specific restriction endonuclease cleavage of salt extracted chromosomes, and their observation could be due to a bias towards larger fragments in their attached DNA from regions of highly reiterative sequence, where it might be expected to find a lower than average frequency of restriction sites. Our data using nonspecific nucleases depends only on the protection afforded the DNA by nonhistone proteins at the presumed sites of attachment to the chromosome backbone.

The different types of protected DNA fragments obtained from metaphase chromosomes depleted of histones either by acid extraction or salt extraction need further study to see what, if any, involvement they have in maintaining the metaphase chromosome structure. In particular, some alternative technique for investigating possibly significant short sequences within the protected fragments is required.

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