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Isolation of a protein scaffold from mitotic HeLa cell chromosomes

(metaphase/nonhistones/DNA superstructure/micrococcal nuclease/dextran sulfate)

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ABSTRACT We have recently shown that, after the histones and most of the nonhistone proteins are gently removed from HeLa metaphase chromosomes, the chromosomal DNA is still highly organized and relatively compact. The structure of these histone-depleted chromosomes is due to the presence of a number of nonhistone proteins that form a central scaffold that retains the approximate size and shape of intact chromosomes and to which the DNA is attached, predominantly forming loops. We now demonstrate that the protein scaffold may be isolated independently of the DNA by treating HeLa chromosomes with micrococcal nuclease before removing the histones.

The chromosomal scaffolds may be isolated by sucrose density gradient centrifugation as a well-defined peak that is stable in 2 M sodium chloride, but is dissociated by treatment with proteases, 4 M urea, or 0.1% sodium dodecyl sulfate. Polyacrylamide gel electrophoresis reveals that the protein content of scaffold preparations is identical to that of histone-depleted chromosomes. Fluorescence microscopy of purified scaffolds in isolation buffer shows that the particles still possess the familiar chromosome morphology. When the scaffolds are examined in the electron microscope, a fibrous structure with the approximate size and shape of intact, paired chromatids is seen. Less than 0.1% of the chromosomal DNA and virtually no histones are associated with the purified scaffold structures.

Mitotic chromosomes are composed of the five histones and a large number of nonhistone proteins that keep the DNA in a highly compact arrangement. Electron microscopy has revealed that the nucleoprotein fiber in mitotic chromosomes is a "knobby" fiber with a diameter of about 200-300 Å and appears in a complex, net-like arrangement (e.g., ref. 1). The complexity of this network and the tendency of the chromatin fibers to adhere to each other make it difficult to study the higher-order folding of the nucleoprotein fiber in chromosomes.

A great deal has been learned in the last few years about the structural role played by the histones in chromatin, but little is known about a possible involvement of nonhistone proteins in chromosome structure. Starting with the hypothesis that nonhistone proteins may play a role in determining the superstructure of interphase and metaphase chromosomes, we have recently developed an approach that permits dissection of the structural contribution made by the histones and certain nonhistone proteins. Our general approach toward uncovering a structural role for the nonhistone proteins has been to gently remove the histones from chromosomes by competition with dextran sulfate and heparin, without destroying the continuity of the DNA fiber (2). In this way, all the histones and many nonhistone proteins are removed, leaving behind some tightly bound nonhistone proteins that keep the chromosomal DNA highly organized and compact. This dissecting procedure has

been successful with both mitotic chromosomes from HeLa cells (2-4) and interphase chromosomes from chick erythroid cells (J. A. Brown, K. W. Adolph, and U. K. Laemmli, unpublished data).

Our studies with mitotic chromosomes are particularly revealing because it was possible to study the histone-depleted chromosomes both biochemically and microscopically (3, 4).

The histone-depleted chromosomes sediment in a sucrose gradient as a broad peak between 4000 and 7000 S. These particles are insensitive to RNase and are stable in 2 M NaCl but are dissociated by mild chymotrypsin treatment or 4 M urea. The histone-depleted chromosomes have a DNA/protein ratio of roughly 6:1, and sodium dodecyl sulfate (NaDodSO₄)/gel electrophoresis reveals 6 major and about 25 minor proteins. Histones are absent at a limit of detection of 0.1% of the original histone content.

The histone-depleted chromosomes have the shape of expanded metaphase chromosomes. In the fluorescence microscope each chromatid is seen to be paired with its sister chromatid and consists of a central structure surrounded by a halo of DNA. The electron microscope reveals these features in greater detail. The micrographs show that the histone-depleted chromosome contains a central structure (scaffold) that extends through each chromatid arm. The DNA is attached to the scaffold transversely, forming loops that originate from adjacent points on the scaffold. It was natural to think that the central scaffold was composed of the residual nonhistone proteins observed by NaDodSO₄/gel electrophoresis.

One might argue, however, that these structures were created during the process of removing the histones and most of the nonhistone proteins, by a rearrangement or trapping of the residual nonhistone proteins. To rule out this possibility it was important to isolate the chromosome scaffold free of the DNA. This was accomplished by treating HeLa chromosomes with micrococcal nuclease prior to depleting the chromosomes of the histones and many nonhistone proteins. Using such a procedure, we have discovered that a nonhistone protein scaffold can be isolated from HeLa metaphase chromosomes. The scaffold retains the general shape and size of intact chromosomes and exhibits a loose, fibrous structure comprised of the same nonhistone proteins associated with histone-depleted chromosomes that have not been treated with nuclease. This paper describes the isolation and preliminary characterization of the chromosomal scaffold.

MATERIALS AND METHODS

Preparation of HeLa Chromosomes and the Chromosome Scaffold. HeLa metaphase chromosomes were prepared according to the method of Wray and Stubblefield (5), in which the cells are kept in metaphase with colchicine and the chro-

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Abbreviations: NaDodSO₄, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.

mosomes are isolated in a buffer containing 1 M hexylene glycol. The chromosomes were purified by differential centrifugation and used within 1 hr of preparation.

The scaffold of HeLa chromosomes was obtained by treating the chromosomes at $A_{260} \sim 10$ with *Staphylococcal* nuclease (Worthington), 40 γ /ml, for 10 min at 0°. The reaction was terminated and the histones, and most nonhistones, were removed by adding the nuclease-treated chromosomes to a solution containing dextran sulfate (2 mg/ml) (Pharmacia), heparin (0.2 mg/ml) (Sigma), 10 mM Tris (pH 9.0), 10 mM EDTA, 0.1% Nonidet P40, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1 volume of chromosomes to 3 volumes of solution. Alternatively, the nuclease-treated chromosomes were diluted 1:1 with a solution such that the following final concentrations were obtained: 2.0 M NaCl, 10 mM Tris (pH 9.0), 10 mM EDTA, 0.1% Nonidet P40, and 1 mM PMSF. After nuclease treatment, this step removes the histones and other proteins that tend to remain associated with the scaffold even after extensive digestion of the DNA.

Sucrose Density Gradient Centrifugation. The scaffold was purified by centrifuging the nuclease-treated and histone-depleted chromosome preparation into 5–60% (wt/vol) linear sucrose gradients containing 0.1 or 2.0 M NaCl, 10 mM Tris (pH 9.0), 10 mM EDTA, 0.1% Ammonyx LO (Onyx Chemical Corp., Jersey City, NJ), and 1 mM PMSF. Centrifugation was in the Beckman SW 50.1 rotor at 4° for 45 min at 3000 rpm with the 0.1 M NaCl sucrose solutions and for 150 min at 6000 rpm with the 2.0 M NaCl solutions. Gradients containing a sucrose concentration from 5 to 60% (wt/vol) were found to be most useful because of the expected heterogeneous size distribution of scaffolds from unfractionated HeLa chromosomes. A sharper peak of radioactivity was found with sucrose density gradients containing 2.0 M NaCl.

Gel Electrophoresis. The sucrose density gradients were collected from the bottom, fractions were pooled, and the proteins (typically labeled with [³⁵S]methionine) were precipitated by diluting the sucrose solutions 1:1 with water and adding trichloroacetic acid to a final concentration of 25% and deoxycholate to a final concentration of 0.125 mg/ml. After 1 hr on ice, the trichloroacetic acid-treated samples were centrifuged to pellet the precipitated proteins, and the pellets were washed with ether or acetone. The pellets were dried and dissolved in final sample buffer (6).

Electrophoresis was carried out with 12.5% polyacrylamide slab gels containing 0.1% NaDodSO₄ by the discontinuous buffer system (6). The gels were evaluated by fluorography (7).

Fluorescence Microscopy. To examine the chromosomal scaffold by fluorescence microscopy, a sample of scaffolds was prepared as described above and purified by sedimentation onto a cover slip through a column of 5% sucrose (wt/vol)/0.1 M NaCl/10 mM Tris, pH 9.0/10 mM EDTA/0.1% Nonidet P40/1 mM PMSF. The scaffolds were stained by adding a drop of the fluorescent protein stain, Mercurochrome, which was used at a 1:1000 dilution. (The Mercurochrome was pharmaceutical grade and came as an aqueous 2% solution.) The HeLa chromosomal scaffold displayed intense fluorescence, suggesting that the scaffold proteins are rich in sulfhydryl groups.

Fluorescence micrographs were taken with a Zeiss Axiomat microscope equipped with an excitation filter system (2X K500, LP455) and a barrier filter (LP 528). The micrographs were recorded with Kodak Tri-X film (ASA 400).

Electron Microscopy. For electron microscopy, chromosomes were treated with *staphylococcal* nuclease as described

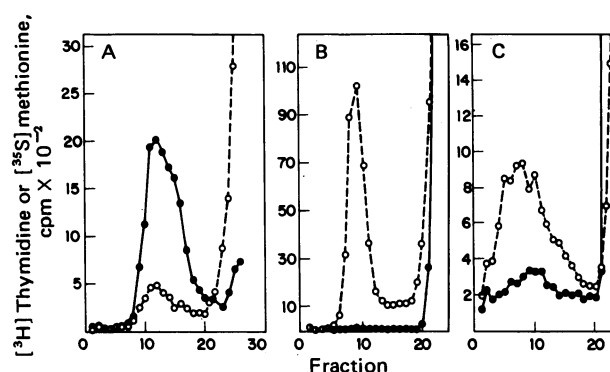


FIG. 1. Isolation of the HeLa chromosome scaffold by sucrose density gradient centrifugation. Chromosomes double-labeled with [³H]thymidine and [³⁵S]methionine were treated with micrococcal nuclease; the histones were removed as described in *Materials and Methods* and layered on 5–60% (wt/vol) sucrose gradients in cellulose nitrate centrifuge tubes of the Beckman SW27 rotor. The direction of sedimentation is from right to left. (A) Control. Histone-depleted chromosomes not treated with nuclease, in a gradient containing 2 M NaCl. (B) Nuclease-treated and histone-depleted chromosomes, in a gradient containing 2 M NaCl. (C) Nuclease-treated and histone-depleted chromosomes, in a gradient containing 0.1 M NaCl. O—O, [³⁵S]methionine; ●—●, [³H]thymidine.

above and then diluted in a solution such that the final concentrations were: 2.0 M NaCl, 10 mM Tris-HCl (pH 9.0), and 10 mM EDTA. After a 10-min incubation at 4°, the sample was diluted 2-fold in 10 mM Tris-HCl, pH 9.0/10 mM EDTA/0.1 M NaCl and spread with cytochrome *c* on a hypophase of 0.125 M ammonium acetate (method to be described elsewhere). Alternatively, scaffolds were treated with 2 M NaCl and purified on a sucrose gradient as described above (except that detergent was omitted from the gradient) and spread as above. Scaffolds were viewed in a Phillips EM300 and photographed at an original magnification of $\times 4000$ – $10,000$.

RESULTS

Isolation of the Chromosomal Scaffold. Chromosomal scaffolds were isolated from metaphase chromosomes exposed to micrococcal nuclease in conditions such that about 60–70% of the DNA was rendered acid-soluble. The chromosomes were subsequently exposed to dextran sulfate and heparin to remove the histones and many nonhistone proteins under exactly the same conditions used for the isolation of histone-depleted chromosomes. Fig. 1 A and B shows the gradient profiles of both histone-depleted chromosomes and scaffolds isolated from chromosomes double-labeled with [³H]thymidine and [³⁵S]methionine. The histone-depleted chromosomes had a sedimentation coefficient ranging between 4000 and 7000 S and about 10% of the [³⁵S]-labeled proteins cosedimented with these structures (Fig. 1A). The chromosomal scaffolds formed a well-defined peak containing, again, about 10% of the total ³⁵S-labeled proteins (Fig. 1B). A small proportion, frequently less than 0.1%, of the [³H]thymidine-labeled DNA cosedimented with the scaffold; the rest of the DNA stayed at the top of the gradient. The residual amount of DNA associated with the scaffolds might represent fragments of DNA that are protected against the nuclease by nonhistone proteins that comprise the scaffold. The amount of DNA associated with the scaffold varied between 1.0% and 0.1% or less in different experiments.

The gradients in Fig. 1 A and B contained 2 M NaCl, demonstrating that the scaffolds are stable in high salt concentrations, as are the histone-depleted chromosomes. The scaffolds

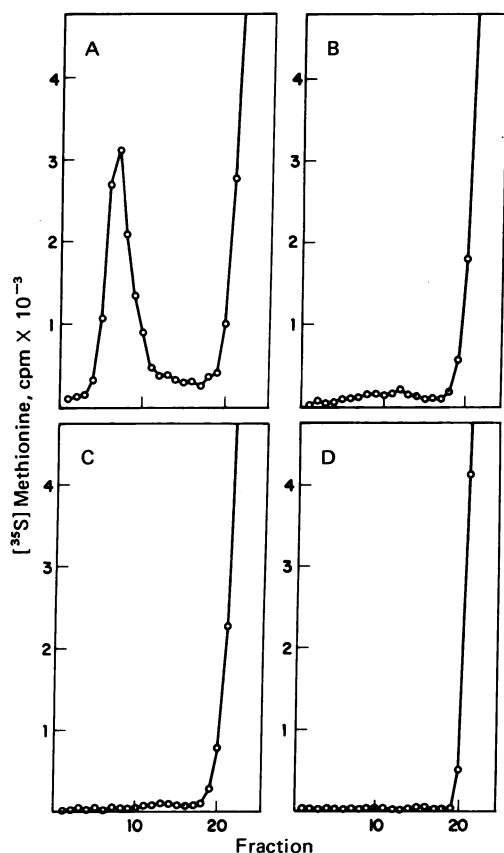


FIG. 2. Dissociation of the HeLa chromosome scaffold. Chromosomes labeled with [^{35}S]methionine were treated with micrococcal nuclease, and the histones were removed by adding the chromosomes to a dextran sulfate/heparin solution containing the various dissociating agents. Similar results were obtained when 2 M NaCl was used instead of dextran sulfate/heparin. The samples were incubated for 30 min at 4° after layering on 5–60% sucrose gradients containing 2 M NaCl in 5-ml centrifuge tubes of the Beckman SW 50.1 rotor. (A) Untreated scaffold control. (B) Pronase at 1 mg/ml. (C) 4 M urea in the sucrose gradient rather than in the dextran sulfate/heparin solution. (D) 0.1% NaDodSO₄.

in the high-salt gradients banded close to their buoyant density equilibrium position and formed a narrow peak for this reason. They formed a broader peak in a gradient containing 0.1 M NaCl (Fig. 1C), which is not unexpected considering the heterogeneity of metaphase chromosomes. They had a sedimentation coefficient roughly 3 to 4 times larger than that of histone-depleted chromosomes, due to the removal of the DNA surrounding the scaffold in the histone-depleted chromosomes.

We have demonstrated elsewhere that the histone-depleted chromosomes are stable in either 0.1 M or 2 M NaCl but are dissociated by proteases, 4 M urea, or 0.1% NaDodSO₄. Fig. 2 shows that the scaffold exhibits the same properties. The peak of the scaffold is missing after treatment of the samples with Pronase, 4 M urea, or 0.1% NaDodSO₄.

Protein Components of Histone-Depleted Chromosomes and Scaffolds. Comparison of the proteins associated with the scaffold with those of histone-depleted chromosomes by NaDodSO₄/gel electrophoresis showed that the major proteins were the same in both structures (Fig. 3). All the minor components that could be detected also were identical. This suggests that few, if any, proteins are associated with the DNA surrounding the scaffold. No histones (that is, much less than 0.1%) could be detected in the scaffold.

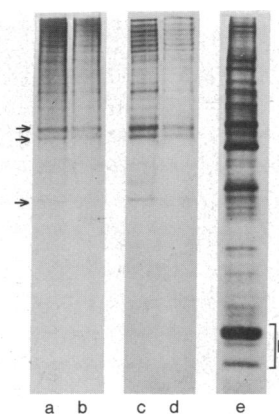


FIG. 3. NaDodSO₄/polyacrylamide gel electrophoresis of the HeLa scaffold proteins. The peak fractions of histone-depleted chromosomes and of the chromosome scaffold were collected from 5–60% sucrose gradients containing 2 M NaCl and prepared for electrophoresis in 12.5% NaDodSO₄/polyacrylamide gels as described in *Materials and Methods*. The direction of migration is from top to bottom. The gels were prepared for fluorography as outlined by Bonner and Laskey (7). Lanes: a and b, peak fractions of histone-depleted chromosomes; c and d, peak fractions of HeLa chromosome scaffolds; e, supernatant from the gradient containing histone-depleted chromosomes, showing the histones and nonhistones that were removed by the dextran sulfate/heparin solution. The arrows indicate the positions of three of the major bands; the bracket "H" shows the mobility region of the nucleosomal histones H2a, H2b, H3, and H4.

Approximately 30 protein bands were revealed in the scaffold by gel electrophoresis. Most of these proteins had molecular weights above 50,000. Because our chromosome preparations were unfractionated with respect to size and because HeLa chromosomes have various types and sizes, it is difficult to conclude whether all of the scaffolds have the same fairly complex protein composition or whether a spectrum of simple structures exist. We think, however, that the former possibility seems more likely.

The pattern of protein bands was similar for scaffold preparations that were purified in sucrose gradients containing 0.1 M and 2.0 M NaCl. Residual traces of histones were more likely to be found with the former. To prepare the scaffold, HeLa chromosomes were first treated with micrococcal nuclease, and then depleted of histones and nonhistones with either a dextran sulfate/heparin solution or with 2.0 M NaCl. To determine whether the method of histone removal influenced the protein composition of the scaffold, NaDodSO₄/polyacrylamide gels were run with scaffolds isolated from sucrose gradients by the dextran sulfate/heparin procedure and by 2.0 M NaCl. The pattern of [^{35}S]methionine-labeled protein bands was the same with both procedures (not shown).

Scaffolds were normally isolated from HeLa chromosomes that had been purified from contaminating cellular debris and nuclei by differential centrifugation. Because it was possible that some of the minor protein bands of the polyacrylamide gel pattern were not true scaffold components but merely had cosedimented with the chromosomes during differential centrifugation, chromosomes were further purified by pelleting through 2.2 M sucrose (8). Scaffold proteins were prepared from such purified chromosomes and subjected to gel electrophoresis. The banding pattern resembled that of chromosomes purified by differential centrifugation, with minor bands still present. Residual contaminating histones were more difficult to eliminate, most likely because the chromosomes tended to aggregate when pelleted through 2.2 M sucrose.

Fluorescence Light Microscopy. The structure of the HeLa

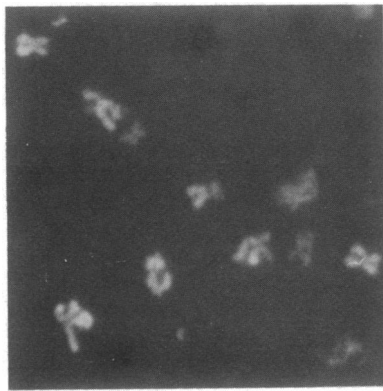


FIG. 4. Fluorescence light microscopy of the scaffold. The scaffolds were prepared for microscopy by centrifugation through a column of 5% sucrose to deposit the scaffolds on a cover slide. A drop of the protein fluorescent stain, Mercurochrome, was added at 1:1000 dilution, and the scaffolds were photographed with the Zeiss microscope. ($\times 1400$.)

chromosome scaffold was investigated with both light and electron microscopy. Light microscopy revealed the general shape of the scaffold while still in the solution used for isolation; electron microscopy presented the possibility of uncovering more detailed aspects of scaffold structure. A representative field showing a number of scaffolds stained with the fluorescent protein stain are presented in Fig. 4. The basic feature of the scaffold seen by fluorescence light microscopy is that the scaffold still retained the general shape and size of chromosomes. In particular, the scaffold particles were dual, probably reflecting the duality of the sister chromatids of the chromosomes, and the particles were connected at one point which most likely represents the centromere. Staining the scaffold with a DNA stain, ethidium bromide, showed that little, if any, of the DNA was still attached.

A detailed study was undertaken to compare the distribution of scaffold sizes in low and high salt concentrations with the sizes of intact chromosomes. The results (Fig. 5) confirm the qualitative observation of the positive correlation between the lengths

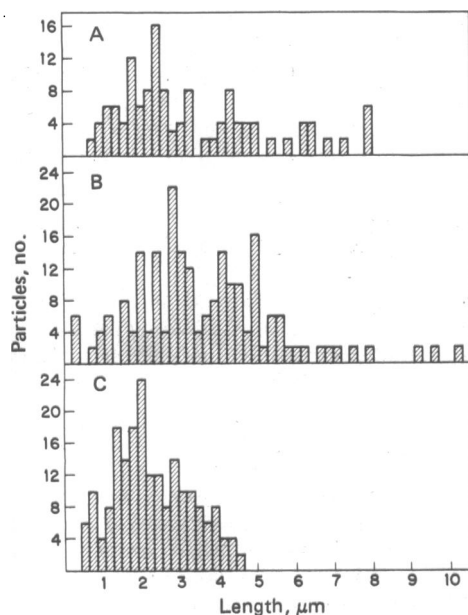


FIG. 5. Histogram of the lengths of purified chromosome scaffolds. (A) Intact chromosomes. (B) Chromosome scaffolds in 0.1 M NaCl. (C) Chromosome scaffolds in 2.0 M NaCl.

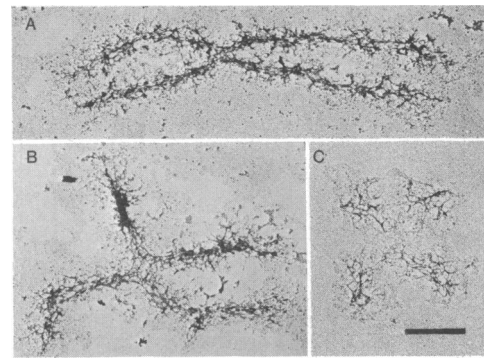


FIG. 6. Electron micrographs of the chromosomal scaffold. (A and B) Chromosomes were treated with micrococcal nuclease and then diluted into 2 M NaCl, incubated at 4°, and spread with cytochrome c. (C) An example of a scaffold purified on a sucrose gradient (see *Materials and Methods*). (Bar represents 2 μ m.)

of the chromosomes and the scaffolds. In 0.1 M NaCl the scaffolds showed a distribution from about 1 to 10 μ m, with the most common lengths being in the range 2–5 μ m. The length of the scaffolds in 2.0 M NaCl was shorter by a factor of about 2. The efficiency of scaffold isolation was the same in low or high salt gradients, suggesting, therefore, that the shorter scaffold distribution is the result of a genuine contraction and not due to the selective loss of longer scaffolds. Most of the scaffolds in 2.0 M NaCl had a length of about 2 μ m and the longest were approximately 5 μ m. The length distribution of the scaffolds in low salt was similar to the distribution of intact HeLa chromosomes in the same solution conditions. The chromosomes showed a broad distribution of lengths from about 1 to 8 μ m with most having lengths between 2 and 5 μ m.

Electron Microscopy. The structure of the HeLa chromosome scaffold revealed by electron microscopy was consistent with the observations made with the light microscope (Fig. 6). The scaffold appeared as a loose, fibrous structure with a length somewhat greater (about 10–15 μ m) than the length of the scaffold viewed by fluorescence microscopy. A regular repeating arrangement of the protein species that constitute the scaffold was not immediately apparent from the electron micrographs of fixed or unfixed scaffolds. The electron micrographs did show that the scaffolds retained the general shape of the chromosomes, displaying a doubleness suggestive of the sister chromatids of the chromosomes and a single connecting point suggesting the centromere region. This is noteworthy because it suggests that the chromatids are either not held together primarily by DNA or, if they are, that DNA must be protected by nonhistones. The fibrous substructure of the scaffold was predominantly oriented in the direction of the major axis of the scaffold, although many projections perpendicular to this orientation were seen in the electron micrographs.

DISCUSSION

We have presented evidence here that a stable structure composed of nonhistone proteins can be isolated from mitotic chromosomes of HeLa cells. This entity, which we call the chromosome scaffold, is identified as the residual structure seen by fluorescence microscopy and electron microscopy at the center of HeLa chromosomes that have been depleted of histones and most of the nonhistones. Electron microscopy has revealed that a large proportion of the DNA is arranged as loops, with contour lengths of at least 10–30 μ m, which extend from closely adjacent points along the scaffold to form a halo sur-

rounding the central scaffold. It thus appears that one level of structural constraint upon the DNA in metaphase chromosomes is provided by the proteins of the scaffold which fold the DNA into loops. This model (scaffolding model) for the structure of metaphase chromosomes has been discussed in greater detail elsewhere (2) and is compatible with the appearance of mitotic chromosomes when the loops of DNA are condensed into tighter structures upon interaction with the histones.

The experiments described in this paper clearly demonstrate that the structure of histone-depleted chromosomes does not result from a rearrangement of the residual nonhistone proteins on the DNA after removal of the histones or from trapping of the nonhistones by a tangle of DNA. Furthermore, the scaffolds are not artifactually produced by the dextran sulfate/heparin procedure, because the histones can be removed, after digestion of the chromosomes with micrococcal nuclease, through the use of 2 M NaCl instead of dextran sulfate/heparin.

A number of questions remain to be resolved concerning the HeLa chromosome scaffold. Of particular interest is the role of the small percentage of DNA, frequently less than 0.1%, that cosediments with the scaffold. We have not treated chromosomes with micrococcal nuclease after removing the histones and most of the nonhistones, and it is possible that the residual fraction of DNA associated with the scaffolds would be digested. However, this small fraction may represent the DNA fragments at the bases of the loops of DNA which are protected against nuclease digestion by the scaffold proteins. A special, possibly highly repeated, sequence might be expected for these fragments if the same protein-DNA interactions are used along the scaffold to constrain the DNA into loops, or at least if a limited number of protein-DNA interactions exist. At present we have little experimental evidence regarding these points.

About 6 major and 25 minor proteins are observed in the scaffold and exactly the same pattern is seen in histone-depleted chromosomes (Fig. 3). This shows that few, if any, proteins are associated with the DNA surrounding the scaffold. The protein constituents of the scaffold are also identical when the histones are removed with either dextran sulfate or 2 M NaCl. Furthermore, isolation of scaffolds in low salt gradients (0.1 M NaCl) or high salt gradients (2 M NaCl) does not alter the protein pattern. It remains to be determined whether all of the scaffold proteins are truly integral components of the scaffold, or whether some of the minor proteins fortuitously cosediment with the scaffold or are weakly bound. The similarity of the gel electrophoresis patterns of the proteins from the scaffold and histone-depleted chromosomes (3) suggests that most of the protein species are true components of the scaffold. It also remains to be determined whether all of the scaffold proteins are structural. In addition to serving as a skeleton that keeps the DNA of metaphase chromosomes in a folded arrangement, the scaffold could possibly also serve, for example, to partition nuclear enzymes into the daughter cells during mitosis. The possibility of such a function for the scaffold could be investigated by testing preparations of scaffolds for enzymatic activity.

Over the years a vast number of observations concerning chromosome structure have been reported in the literature and a large number of chromosome models have been presented. A recent model that incorporated the concept of a chromosome "core" was presented by Stubblefield and Wray (9), based on their electron microscope studies of Chinese hamster metaphase chromosomes subjected to shearing forces and extraction with

2 M NaCl and 6 M urea. Their model proposed that chromosomes consist of two ribbons of many parallel nucleoprotein fibers wound into a "core" containing 25% of the DNA, and that "epichromatin" loops are attached along the length of the "core." The major difficulty in interpreting their electron micrographs comes from the tendency of the nucleoprotein fibers to adhere to each other and become longitudinally oriented as a result of stretching. Our work shows that the scaffold is an independent entity and biochemically distinct from the rest of the chromosome. In an earlier paper, Maio and Schildkraut (8) briefly mentioned that, after exhaustive treatment of chromosomes with pancreatic DNase and RNase and extraction of the histones with 0.2 M HCl, faint, fibrous structures that resembled metaphase chromosomes could still be seen by phase contrast microscopy.

It is also possible that the scaffold may play a more dynamic role in condensing the relatively diffuse interphase chromatin to form mitotic chromosomes. Although to propose such an active role for the scaffold must remain pure speculation at present, a major goal of future studies will be to detail the contribution of nonhistone proteins in both interphase and metaphase cells and to determine the dynamic relationship between the two states. As a step in this direction, recent preliminary experiments with interphase HeLa nuclei show that the major scaffold proteins are present in interphase nuclei. More detailed studies of this type will go a long way toward elucidating the macromolecular interactions that are responsible for the state of the eukaryotic genome through the cell cycle and contribute to a more complete understanding of the structure of metaphase chromosomes.

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