ISOLATION OF METAPHASE CHROMOSOMES FROM HELA CELLS

JOEL A. HUBERMAN and GIUSEPPE ATTARDI

From the Division of Biology, California Institute of Technology, Pasadena

ABSTRACT

The authors have developed a method for large-scale isolation of metaphase chromosomes from HeLa cells. The distinguishing feature of this method is the use of a pH sufficiently low (about 3) to stabilize the chromosomes against mechanical damage. Many milligrams of fairly pure, morphologically intact chromosomes can be isolated in 8 hr or less of total working time. The isolated chromosomes contain about 2.0 mg of acid-soluble protein, 2.7 mg of acid-insoluble protein and 0.66 mg of RNA for each milligram of DNA. The RNA bound to the isolated chromosomes consists mainly of ribosomal RNA, but there is also a significant amount of 45S RNA.

INTRODUCTION

Many possible biochemical and biophysical approaches to the study of chromosomes in higher organisms have been hindered, until recently, by the lack of suitable procedures for large-scale isolation of chromosomes. Although the methods for isolation of interphase chromosomes, or "chromatin," which have been developed in recent years (1, 2) are satisfactory for certain purposes, a definite need still exists for a procedure which will allow large-scale isolation of morphologically intact metaphase chromosomes. Metaphase chromosomes are an indispensable complement to interphase chromosomes for the general study of chromosome structure. In addition, metaphase chromosomes have the unique advantage of being so condensed that they can be distinguished microscopically both from each other and from contaminating nonchromosomal material. Consequently, one is not limited to studying the average properties of all chromosomes; one can also examine single types of chromosomes.

According to our experience, in the isolation of metaphase chromosomes by most previously published methods (3–5), morphological damage to some of the chromosomes cannot be avoided and only partial purification of the chromosomes from cell debris can be achieved. We report here a method for the rapid preparation, in milligram quantities, of fairly pure, morphologically intact metaphase chromosomes from HeLa cells. We also report the results of studies on the chemical composition of isolated chromosomes.

MATERIAL AND METHODS

Cultivation of Cells

HeLa S3 cells (6) were grown in suspension culture in a modified Eagle's medium (7) supplemented with 5% calf serum. For accumulation of metaphase cells, partial synchrony was induced by lowering the culture temperature to 4°C for 1 hr and then returning it to 37°C (8). Ten to 11 hr later, colchicine was added to a final concentration of 0.5 to 1×10^{-5} M. The cells were harvested by centrifugation 9 to 10 hr after colchicine addition and washed 3 times in 0.137 M NaCl, 0.005 M KCl, 0.007 M NaH_2PO_4, 0.025 M Tris, pH 7.4. This procedure routinely produced about 30% metaphase cells.

Isolation of Chromosomes

All operations were carried out in the cold (0° to 4°C). The pellet of washed cells was gently resuspended in 15 vol of 0.1 M sucrose, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl_2$ (4). The cells swelled in this hypotonic medium and the chromosomes in metaphase cells became excellently separated from each other. Five min later, 3 vol of 0.1 M sucrose, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl_2$, 3.3×10^{-3} M HCl were added slowly, with stirring, to each volume of cell suspension. Slow addition of the acid solution was necessary to prevent clumping of the chromosomes in metaphase cells. The measured final pH was about 3.0. Higher pH values (up to 3.3) allowed satisfactory breakage of cells and conservation of chromosome morphology, but separation of the chromosomes from cytoplasmic debris was more difficult.

A phase-contrast microscope was used to check the result of acid addition. Cells suspended in hypotonic medium appeared grey, with little internal contrast. The chromosomes in metaphase cells were barely visible. After the pH had been adjusted to 3.3–3.0, the chromosomes, evenly distributed throughout the cytoplasm of metaphase cells, appeared distinct and bright.

After adjustment of pH, a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle was used to homogenize the cells. The course of homogenization was checked with a microscope. As an end point for homogenization, the time was chosen when all interphase cells were broken (usually after less than 1 min). At this stage the great majority of metaphase cells were also broken.

The released chromosomes were usually single and free of obvious attached debris. The following steps separated these chromosomes from the nuclei and cytoplasmic debris which were also produced by homogenization.

The homogenate was centrifuged at 900 *g* (2000 RPM in the International PR2 centrifuge, head No. 269, International Equipment Co., Needham Heights, Massachusetts) for 30 min. The resulting pellet contained nuclei, chromosomes, and the larger cytoplasmic debris. Most debris remained in the supernatant.

The supernatant was discarded and the pellet resuspended in HCM (1×10^{-3} M HCl, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl)_2$, using about 40 ml of HCM for each milliliter of pellet. The suspension was rehomogenized briefly with a Potter-Elvehjem homogenizer to break up any clumps that might have formed as a result of pelleting.

Up to 20 ml of suspension at a time were then gently layered onto 200 ml of a 0.1 to 0.8 M linear sucrose gradient in HCM (final pH adjusted to 3.0) which had been formed in a 250 ml glass centrifuge bottle. The gradient was accelerated at 500 RPM per min to 1500 RPM (450 *g*) in the International PR-2 centrifuge, head No. 284, and held at that speed for 20 min. Deceleration was also at 500 RPM per min. After the centrifugation the chromosomes were distributed from near the bottom of the gradient to near the top. Cytoplasmic debris remained at or near the top, extending into the chromosome region. Nuclei and some clustered chromosomes were pelleted at the bottom. A crude fractionation of chromosomes on the basis of sedimentation velocity was also produced; most large chromosomes were found near the bottom, while most small chromosomes remained near the top.

The top 20 ml of the gradient were discarded and the rest was sucked off, leaving a small amount (about 10 ml) in the bottom of the centrifuge bottle so as not to disturb the pelleted nuclei. The supernatant was then mixed until the sucrose was evenly distributed, and the chromosomes were collected by centrifugation at 850 *g* (2000 RPM in the International PR-2 centrifuge, head No. 284) for 90 min. The pellet contained very few nuclei (less than 3% of the total DNA in the pellet was from whole nuclei if the initial proportion of metaphase cells was 15% or greater). There was, however, still considerable contamination by debris.

Most of the debris was removed by the following procedure. The pellet was resuspended in a small volume of HCM with brief rehomogenization to break up clumps. Ten ml of 2.2 M sucrose in HCM were placed in a Spinco SW-25 plastic tube (Beckman Instruments, Inc., Palo Alto, California) and 15 to 20 ml of chromosome suspension were layered on top. The upper three-fourths of the tube contents were gently stirred to form a rough gradient. After centrifugation at 20,000 RPM for 1 hr the chromosomes were found in a pellet at the bottom of the tube, while most cytoplasmic debris remained floating above the 2.2 M sucrose layer. The yield of chromosomes at this point, as determined by DNA determination (see below) or by direct counting in a Petroff-Hausser counting chamber (C. A. Hausser and Son, Philadelphia, Pennsylvania), was about one-third of the chromosomes from all cells scored as in metaphase before homogenization.

Chromosome Storage

Chromosomes stored in HCM at 2° to 4°C retained their morphological integrity for many months. They could also be stored frozen in HCM at −70°C.

Chemical Analysis

Acid-soluble proteins were extracted from chromosomal or nuclear suspensions with 0.2 M HCl at 0°C for ½ hr. The residue was removed by centrifugation and extracted once more with another portion of

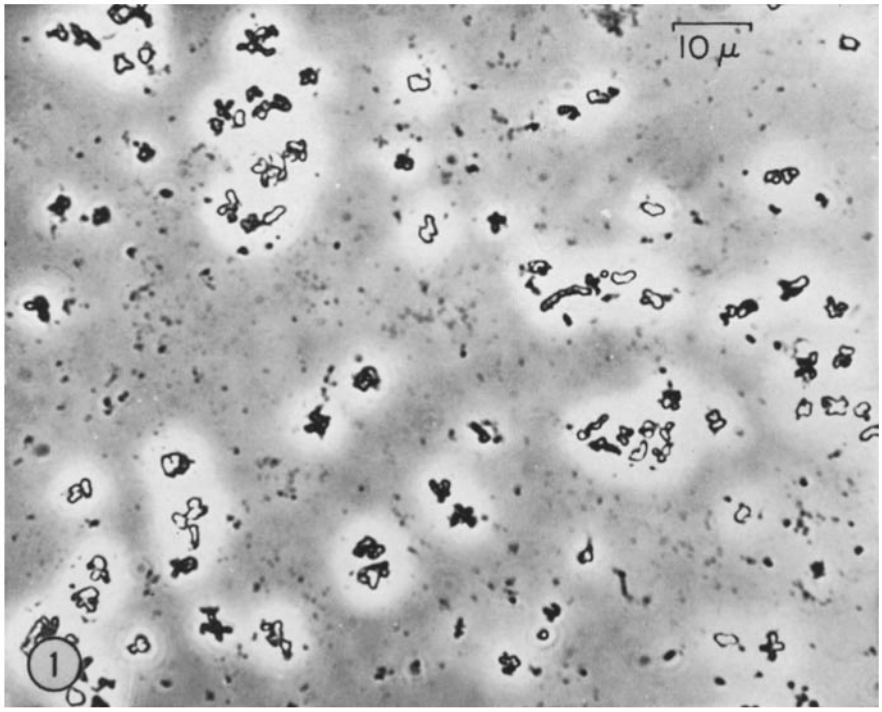

FIGURE 1 Isolated HeLa metaphase chromosomes suspended in HCM. Phase contrast. × 1100.

0.2 M HCl. Trichloroacetic acid was added to the pooled supernatants to a final concentration of 20%. The acid-soluble proteins were allowed to precipitate overnight at 0°C and were then collected by centrifugation, dissolved in 1 M NaOH, and determined by the method of Lowry et al. (9). Vacuum-dried calf thymus histone was used as a standard.

The residue left after HCl extraction was washed once with ethanol-ether (3:1), then resuspended in 10% trichloroacetic acid and heated at 100°C for 20 min to hydrolyze nucleic acids. After one more wash with 10% trichloroacetic acid the residue was dissolved in 1 M NaOH, and acid-insoluble proteins were determined by the method of Lowry et al. (9) using vacuum-dried bovine serum albumin as a standard.

For nucleic acid determinations, the general procedure of Schmidt and Thannhauser (10) was followed. Chromosomal or nuclear suspensions were precipitated with 10% trichloroacetic acid, washed once with ethanol ether (3:1), then dissolved in 0.3 M KOH. RNA was hydrolyzed by incubation at 37°C for 18 hr. Perchloric acid was then added to a final concentration of 0.5 M, and the samples were kept at 0°C for at least ½ hr. The precipitate of DNA, protein, $KClO_4$, and other materials was washed once with a small volume of 0.5 M perchloric acid. The wash was combined with the RNA hydrolysate, and RNA in this pooled solution was determined by the orcinol method (11) using D-ribose as a standard.

DNA in the precipitate was determined, after hydrolysis in 0.5 M perchloric acid at 70°C for 15 min, by the diphenylamine procedure as described by Burton (12), using D-deoxyribose as a standard.

RNA Purification

RNA was purified from isolated chromosomes or nuclei by a procedure described in detail elsewhere (13) which involves cold phenol–sodium dodecylsulfate extraction of total nucleic acids, followed by digestion of DNA with RNase-free DNase.

Acridine Orange Staining

Samples were air-dried on clean glass slides, fixed in 95% ethanol-ether (1:1) and stained according to the procedure of von Bertalanffy et al. (14). A Zeiss fluorescence microscope equipped with an HBO 200W mercury light source, a Schott BG12 excitation filter, and an Sp Orange 2 barrier filter was used to examine the slides.

TABLE I

Base Composition of HeLa Chromosomal and Whole HeLa Cell DNA

Each number represents the average of values obtained from two separate aliquots of the same hydrolysate. Chromosomal DNA was prepared from chromosomes which had been held at pH 3 between 0° and 4°C for 12 hr.

	Mole %					
	T	C	A	G	% GC	Pu/Pyr
Exp. 1						
Chromosomal	30.0	20.0	29.3	20.7	40.7	1.00
Whole cell	30.2	20.1	29.5	20.2	40.3	0.99
Exp. 2						
Chromosomal	30.0	20.0	30.1	19.9	39.9	1.00
Whole cell	30.1	19.9	30.1	19.9	39.8	1.00

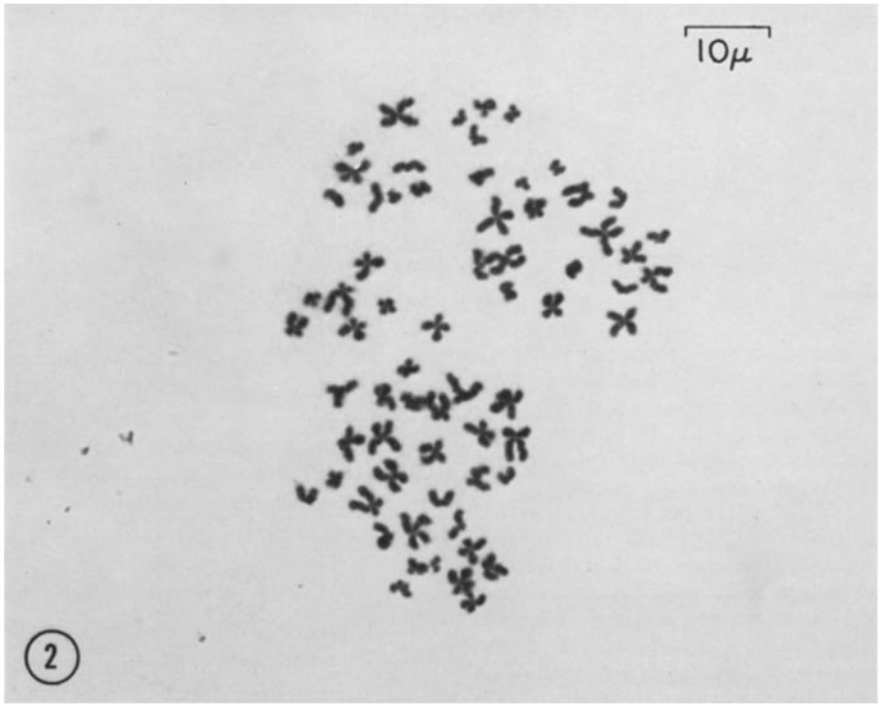

FIGURE 2 The metaphase chromosomes of a single HeLa cell. Bright field. Cells were blocked in metaphase with colchicine, suspended in 1% sodium citrate for 10 min, fixed in acetic acid–ethanol (3:2) for 10 min and then stained in 1% orcein in lactic acid–acetic acid (1:1). Cells suspended in stain solution were squashed by thumb pressure between a slide and a cover slip. × 1100.

Base Composition

DNA was purified from isolated chromosomes or from whole HeLa cells by the Marmur procedure (15). About 400 μg of DNA were dissolved in 0.5 ml of 88 to 90% formic acid and hydrolyzed in a sealed tube under nitrogen at 175°C for 1 hr (16). The hydrolysate was evaporated to dryness and redissolved in 25 μl of 1 M HCl. Two 10 μl portions were used for chromatography. Descending chromatography was carried out on Whatman No. 1 filter paper, using methanol:concentrated HCl:H_2O

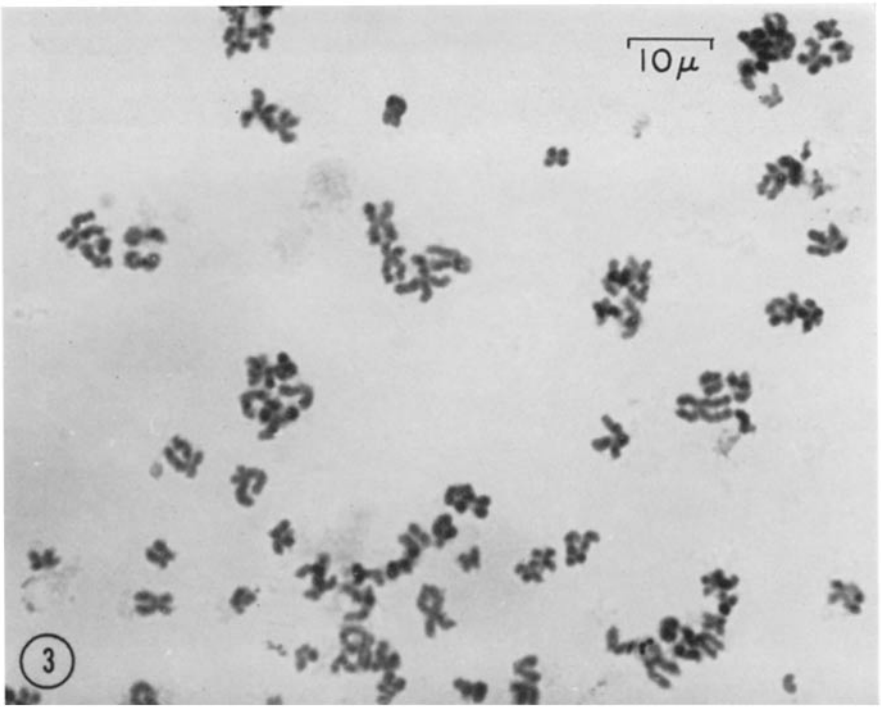

FIGURE 3 Isolated HeLa metaphase chromosomes. Bright field. A small quantity of chromosome suspension in HCM was spread on a glass slide and allowed to dry. The slide was treated with 1% sodium citrate for 10 min, fixed in acetic acid–ethanol (3:2) for 10 min and then stained in 1% orcein in lactic acid–acetic acid (1:1). × 1100.

(70:20:10 by vol) as solvent (17). The chromatograms were dried, and the bases were located with a short wavelength UV light. The bases were eluted in small volumes of 0.1 M HCl and determined spectrophotometrically. The extinction coefficients given by Bendich (18) were used.

RESULTS AND DISCUSSION

Effects of Low pH

A distinguishing feature of the chromosome isolation procedure presented here is the use of a pH sufficiently low (about 3) to stabilize the chromosomes against mechanical damage and to weaken the cytoplasm so that the cells break easily and aggregation of cytoplasmic debris is minimized. Low pH (30% acetic acid; pH 1.8) has also been used by Somers et al. (4) for chromosome isolation. However, under their conditions histones were completely extracted. A third isolation method employing low pH (pH 3.7) has recently been reported (19).

Lowering the pH has the effect of increasing the contraction of the chromosomes. As viewed in the phase-contrast microscope, the chromosomes become smaller and also brighter. The bright appearance of acid-treated chromosomes is evident in Fig. 1. It is caused by an increase in the refractive index of the chromosomes as they contract. This extreme contraction is partly responsible for the increased resistance of the chromosomes to mechanical damage at low pH. However, contraction alone cannot completely explain low pH stabilization: although chromosomes can be made to contract equally well at higher pH (5–7) by the use of sufficiently large (ca. 3×10^{-3} M) concentrations of divalent cations, they still remain susceptible to mechanical damage. The unique strengthening achieved at low pH may be a result of the denaturation and precipitation of some chromosomal proteins.

Low pH was also found to be critical for successful liberation of chromosomes from metaphase cells. At pH values higher than about 3.3, chromo-

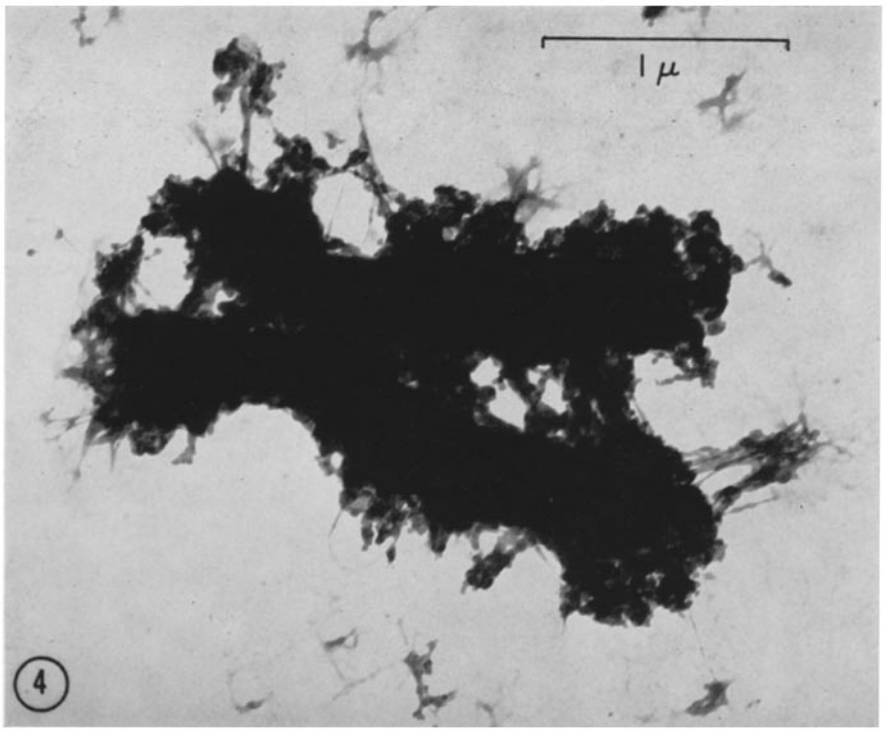

FIGURE 4 Electron micrograph of a typical isolated HeLa metaphase chromosome. Grids were prepared by touching the carbon-Formvar film to the surface of a suspension of chromosomes in HCM, then loading immediately into a grid holder under 30% ethanol. The rest of the procedure has been described by DuPraw (21). (Courtesy of Dr. E. J. DuPraw.) × 33,000.

somes were only partially released during homogenization, and they tended to aggregate with cytoplasmic debris during pelleting.

The use of such a low pH introduces the possibility of undesirable side effects. Certainly, low pH causes denaturation of some chromosomal proteins, but this would not be a drawback for most applications of isolated chromosomes. Low pH might also extract histones. This possibility has been examined, and it has been found that most histones are not extracted under the conditions of our isolation procedure (20). However, some lysine-rich histones found in samples of HeLa chromatin prepared without use of low pH are extracted (20).

In addition, low pH might cause depurinization of nucleic acids. To test this possibility, we determined the base composition of DNA purified from isolated chromosomes and compared it with the base composition of DNA purified from whole HeLa cells. The results are presented in Table I. No loss of purines was detected in chromosomal DNA. If depurinization occurs, it must be less extensive than the experimental error, estimated to be about 1%.

Morphology and Purity of Isolated Chromosomes

The metaphase chromosomes from a typical colchicine-treated HeLa cell prepared by the standard squash technique are shown in Fig. 2. They should be compared to the isolated chromosomes shown in Fig. 3. It is evident that the isolated chromosomes are very similar to the chromosomes prepared by the standard squash technique. Indeed, when the pH was kept below 3.3, we found no examples of morphological distortion during isolation.

Dr. E. J. DuPraw has been kind enough to examine our isolated chromosomes with the electron microscope, using his whole-mount technique

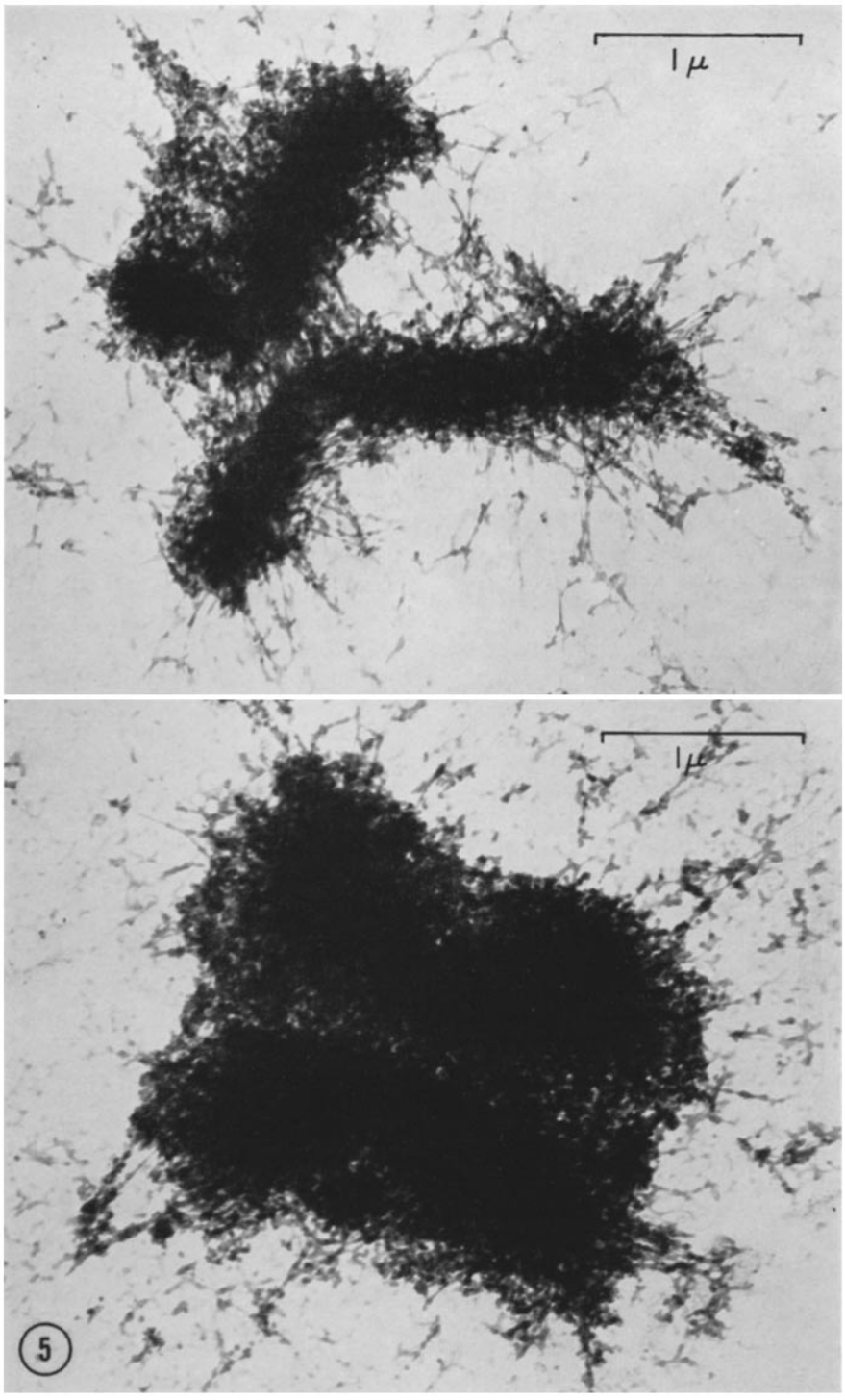

FIGURE 5 Electron micrographs of well preserved, isolated HeLa metaphase chromosomes. Grids were prepared as in Fig. 4. (Courtesy of Dr. E. J. DuPraw.) × 28,000.

TABLE II

Chemical Composition of Isolated HeLa Chromosomes, Nuclei, and Chromatin

Each value for chromosomes and nuclei represents the average of triplicate determinations on each of four separate preparations. Each value for chromatin represents the average of triplicate determinations on one preparation. Chromosomes were isolated as described in the Materials and Methods section. Interphase nuclei were isolated from the same cell homogenates used in chromosome preparations. The nuclear pellet from the sucrose gradient centrifugation was collected and freed from any contaminating cytoplasm by centrifugation through 2.2 M sucrose (in the same manner as chromosomes). Chromatin was isolated from whole HeLa cells (1, 20).

	mg RNA / mg DNA	mg acid-soluble protein / mg DNA	mg acid-insoluble protein / mg DNA
Chromosomes	0.66	2.0	2.7
Nuclei	0.38	1.9	2.1
Chromatin	0.15	1.1	1.0

(21). He found that typical isolated chromosomes had the extremely condensed appearance shown in Fig. 4. The thin fibers, which he has found in honey bee (21) and human (22) chromosomes, if present, seemed fused together. However, in a small proportion of isolated chromosomes, such thin fibers could be readily observed (Fig. 5). The chromosomes used for these pictures were suspended in HCM. The "fusion" of fibers evident in Fig. 4 is probably the manifestation, at the electron microscope level, of the extreme chromosome contraction observed in HCM at the light microscope level. However, the contraction observed in HCM has been found to be a reversible phenomenon. All isolated chromosomes are capable of expanding at the light microscope level. For example, the chromosomes in Fig. 3 have been expanded (relative to those in Fig. 1) by the treatment described in the legend to Fig. 3. It is possible that all expanded, isolated chromosomes would reveal fibers like those in Fig. 5.

In the absence of reliable information on the chemical composition of metaphase chromosomes (see below), purity of the chromosome preparations must also be determined morphologically. Unfortunately the morphological criterion is not a quantitative one. Some contamination by cytoplasmic or nuclear debris certainly does remain in our preparations. However, we cannot say how much. The greyish flecks visible in the background of Fig. 1 are contaminating debris. A better estimate of the extent of RNA- or DNA-containing contamination can be made by using acridine orange staining and fluorescence microscopy. After acridine orange staining, red-fluorescing cytoplasm shows a sharp contrast to the yellow-green-fluorescing chromosomes. When this method is applied to our isolated chromosome preparations, a small amount of RNA-containing contamination in the form of isolated debris or of bodies apparently attached to the chromosomes can be recognized. DNA-containing debris is not apparent, however.

Chemical Composition of Isolated Chromosomes

Despite the presence of a certain amount of contamination in our chromosome preparations, we felt that a chemical composition study would be valuable, both to provide an indication of the actual chemical composition of purified chromosomes and as a reference for further chromosome purification. We have also studied the chemical composition of whole interphase HeLa nuclei and interphase HeLa chromatin. Our results are presented in Table II.

The large amount of RNA in metaphase chromosomes relative to interphase chromatin and even to whole nuclei suggests, at first, that cytoplasmic contamination may be extensive. There are several reasons, however, for thinking that the RNA content of metaphase chromosomes may really be unusually large. First, we have some evidence that a large fraction of the RNA in our chromosome preparations is actually bound to the chromosomes; isolated chromosomes which have been extensively pretreated with DNase fluoresce orange-red rather than yellow-green after acridine orange staining. The amount of red staining due to chromosomes after DNase treatment seems, by visual estimate, to be considerably greater than that due to debris. Subsequent RNase treatment shows that the red staining of DNase-treated chromosomes (and of debris) is probably due to RNA and not to denatured DNA; only a barely visible greenish fluorescence remains.

Second, cytological studies (23–26) have shown that during the course of mitosis the amount of

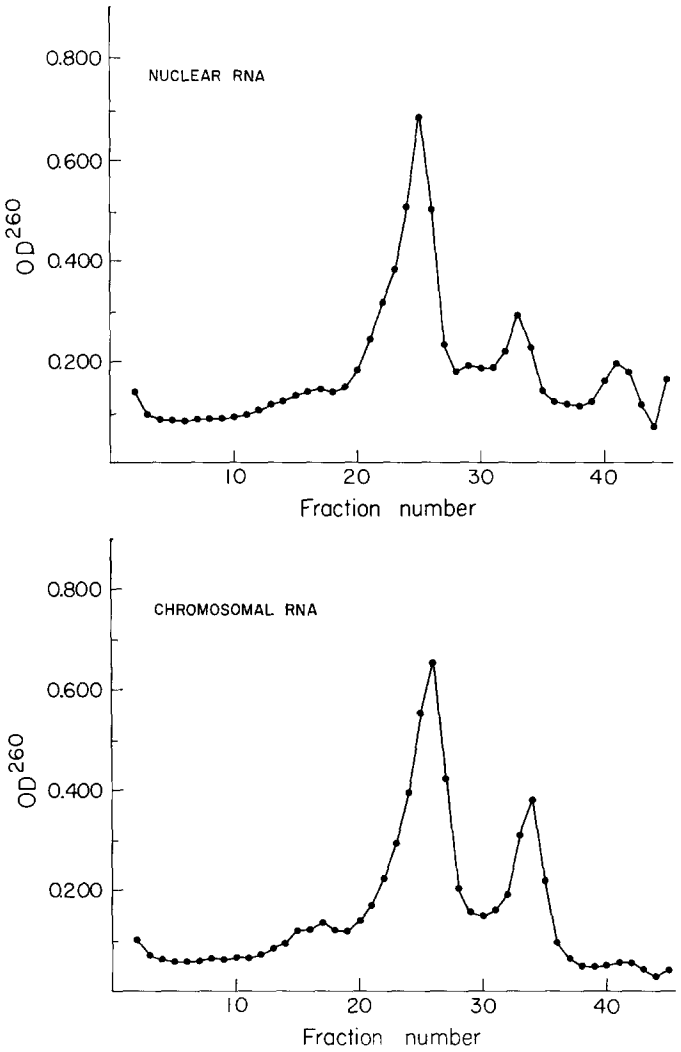

FIGURE 6 RNA was purified (as described in the Materials and Methods section) from a quantity of isolated chromosomes containing about 0.5 mg of DNA and from a quantity of nuclei, isolated as described in Table II, containing about 1.5 mg of DNA. The RNA was dissolved in 0.5 ml of acetate buffer (0.1 M NaCl, 0.01 M sodium acetate buffer, pH 5.0) and layered on top of 25 ml linear 5 to 20% sucrose gradients in the same buffer. The gradients were centrifuged at 25,000 RPM at 2°C in the Spinco Model L ultracentrifuge for 7 hr.

RNA bound to the chromosomes increases, reaching a maximum at metaphase; it then gradually decreases during anaphase and telophase. These changes in chromosomal RNA content during mitosis have been termed the "chromosomal RNA cycle" (27).

Finally, investigators in other laboratories, using metaphase chromosomes isolated by different procedures, have also found very high RNA contents in metaphase chromosomes. Lin and Chargaff (5) have found an RNA to DNA ratio of 0.64 for HeLa metaphase chromosomes, while Cantor and Hearst (19) have reported an RNA to DNA ratio of 1.0 for mouse ascites tumor metaphase chromosomes. Maio and Schildkraut, in a recently published abstract (28), have reported an RNA to DNA ratio of 0.8 for HeLa metaphase chromosomes.

Our findings for the protein content of metaphase chromosomes also require comment. First,

our acid-soluble proteins should not be considered equivalent to histones. As pointed out above, some lysine-rich histones are lost during preparation. Also, many nonhistone proteins are known to be acid-soluble (1). Thus no significance can be given, at the present time, to the greater proportion of acid-soluble proteins in metaphase chromosomes than in interphase chromatin. The protein results may also be misleading because of the unknown extent of contamination and because of variation in the color values for different proteins in the test of Lowry et al. (9).

Sedimentation Profile of RNA from Isolated Chromosomes

We have taken a first step toward elucidation of the nature of the RNA bound to metaphase chromosomes by purifying RNA from isolated metaphase chromosomes and comparing it to RNA from interphase nuclei. The sedimentation profile of RNA from these sources is shown in Fig. 6. The sedimentation profile of HeLa nuclear RNA is similar to that found by Penman (29) for the same material, and by Steele et al. (30) for rat liver nuclear RNA. One recognizes two peaks, corresponding to the two ribosomal RNA species, and a faster component with a sedimentation constant of about 45S. The latter presumably represents the large size ribosomal RNA precursor described in different types of animal cells (31–33). The presence in the nucleus of 18S RNA in amounts considerably smaller, relative to the major ribosomal RNA component, than found in cytoplasmic ribosomal RNA is in agreement with Penman's observations (29), suggesting that there are no mature ribosomes, but only precursors, in the nucleus: according to this author, the 45S RNA is cleaved into 18S RNA, which is immediately transferred to the cytoplasm, and 35S RNA, which remains in the nucleus to be transformed into 28S RNA. In addition to the ribosomal RNA species and their large precursors, one can see in the sedimentation profile of nuclear RNA small amounts of 4S RNA, and a polydisperse RNA with sedimentation constants between 6S and more than 50S. The latter material presumably represents, at least in part, the heterogeneous nonribosomal type nuclear RNA described in HeLa cells (34) and other animal cells (13, 35, 36).

The sedimentation profile of the RNA extracted from metaphase chromosomes also shows the two ribosomal RNA components and the 45S RNA species. The amount of ribosomal RNA relative to DNA is about three times as large as in nuclear RNA; there is, on the contrary, relatively less polydisperse RNA and only a very small amount of 4S RNA. As concerns the significance and origin of the chromosomal associated RNA, only speculations are possible at present. Evidence has been presented that ribosomal RNA precursors are localized in the nucleoli (30, 31). Hence the presence of a 45S component in chromosomal RNA is consistent with the hypothesis that, during prophase, at least some of the materials from the disintegrating nucleoli are bound to the condensing chromosomes. More difficult to interpret is the presence of the two ribosomal RNA species. The fact that the ratio of major to minor component is similar to that observed in cytoplasmic ribosomal RNA may be indicative of a cytoplasmic origin for these species (either as a result of accidental contamination during extraction or of an association of physiological significance occurring during mitosis). On the other hand, one cannot exclude the possibility that some of these ribosomal components were still intranuclear at the end of prophase and became associated with the condensing chromosomes. Further experiments will be required to determine the origin and significance of the ribosomal RNA present in the preparations of metaphase chromosomes.

This work was supported by United States Public Health Service grants GM-11726 and 5-Fl-GM-21,622.

The authors gratefully acknowledge the help of Mr. John Elberfeld in part of this work and the valuable technical assistance of Mrs. Benneta Keeley and Mrs. LaVerne Wenzel.

Received for publication 4 April 1966.

REFERENCES

1. Bonner, J., Chalkley, R. G., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Widholm, J., *Method Enzymol.* in press.
2. Frenster, J. H., Allfrey, V. G., and Mirsky, A. E., *Proc. Nat. Acad. Sc.*, 1963, **50**, 1026.
3. Chorąży, M., Bendich, A., Borenfreund, E., and Hutchison, D. J., *J. Cell Biol.*, 1963, **19**, 59.

4. SOMERS, C. E., COLE, A., and HSU, T. C., *Exp. Cell Research, Suppl. 9*, 1963, 220.
5. LIN, H. J., and CHARGAFF, E., *Biochim. et Biophysica Acta*, 1964, **91**, 691.
6. PUCK, T. T., and FISHER, H. W., *J. Exp. Med.*, 1956, **104**, 427.
7. LEVINTOW, L., and DARNELL, J. E., *J. Biol. Chem.*, 1960, **235**, 70.
8. NEWTON, A. A., *in* Synchrony in Cell Division and Growth, (E. Zeuthen, editor), New York, Interscience Publishers, Inc., 1964, 441.
9. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
10. SCHMIDT, G., and THANNHAUSER, S. J., *J. Biol. Chem.*, 1945, **161**, 83.
11. SCHNEIDER, W. C., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press Inc., 1957, **3**, 680.
12. BURTON, K., *Biochem. J.*, 1956, **62**, 315.
13. ATTARDI, G., PARNAS, H., HWANG, M. H., and ATTARDI, B., *J. Mol. Biol.*, in press.
14. VON BERTALANFFY, L., MASIN, M., and MASIN, F., *Cancer*, 1958, **11**, 873.
15. MARMUR, J., *J. Mol. Biol.*, 1961, **3**, 208.
16. WYATT, G. R., *in* The Nucleic Acids, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press Inc., 1955, **1**, 243.
17. KIRBY, K. S., *Biochim. et Biophysica Acta*, 1955, **18**, 575.
18. BENDICH, A., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press Inc., 1957, **3**, 715.
19. CANTOR, K. P., and HEARST, J. E., *Proc. Nat. Acad. Sc.*, 1966, **55**, 642.
20. HUBERMAN, J. A., FAMBROUGH, D. M., DAHMUS, M., and SADGOPAL, A., in preparation.
21. DUPRAW, E. J., *Proc. Nat. Acad. Sc.*, 1965, **53**, 161.
22. DUPRAW, E. J., *Nature*, 1966, **209**, 577.
23. KAUFMANN, B. P., MCDONALD, M., and GAY, H., *Nature*, 1948, **162**, 814.
24. JACOBSON, W., and WEBB, M., *Exp. Cell Research*, 1952, **3**, 163.
25. BOSS, J., *Exp. Cell Research*, 1955, **8**, 181.
26. LOVE, R., *Nature*, 1957, **180**, 1338.
27. MAZIA, D., *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press Inc., 1961, **3**, 181.
28. MAIO, J. J., and SCHILDKRAUT, C. L., *Fed. Proc.*, 1966, **25**, 707.
29. PENMAN, S., *J. Mol. Biol.*, 1966, **17**, 117.
30. STEELE, W. J., OKAMURA, N., and BUSCH, H., *J. Biol. Chem.*, 1965, **240**, 1742.
31. PERRY, R. P., *Proc. Nat. Acad. Sc.*, 1962, **48**, 2179.
32. SCHERRER, K., LATHAM, H., and DARNELL, J. E., *Proc. Nat. Acad. Sc.*, 1963, **49**, 240.
33. GEORGIEV, G. P., SAMARINA, O. P., LERMAN, M. J., SMIRNOV, M. N., and SEVERTZOV, A. N., *Nature*, 1963, **200**, 1291.
34. HOUSSAIS, J. F., and ATTARDI, G., *Proc. Nat. Acad. Sc.*, in press.
35. SIBATANI, A., DE KLOET, S. R., ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1962, **48**, 471.
36. BRAWERMAN, G., GOLD, L., and EISENSTADT, J., *Proc. Nat. Acad. Sc.*, 1963, **50**, 630.