STUDIES ON THE STRUCTURE AND FUNCTION OF MAMMALIAN CHROMOSOMES

thesis by

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This thesis is dedicated

to my parents

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ABSTRACT

At levels of organization between the Watson-Crick model of DNA on the one hand, and the microscopically visible mitotic or meiotic chromosome on the other, very little is known about the structure or function of chromosomes in eukaryotic organisms. The studies reported in this thesis were an attempt to learn more about the arrangement of certain DNA sequences in mammalian chromosomes, about the size of the DNA molecules in such chromosomes, and about the replication of these DNA molecules.

Part I contains the results of experiments designed to determine the distribution of the hundreds of genes (DNA sequences) for ribosomal RNA among the chromosomes of HeLa cells. In the course of these experiments, methods were developed for isolating metaphase chromosomes on a large scale from HeLa cells and for fractionating them on the basis of sedimentation velocity. Hybridization experiments between ribosomal RNA and DNA from the various fractions of isolated chromosomes showed that the genes for ribosomal RNA are confined entirely to small HeLa cell chromosomes.

In Part II are reported the results of autoradiographic experiments intended to help determine the size and manner of replication of the DNA in mammalian chromosomes. All the experiments described in Part II are the result of collaboration with Dr. Arthur D. Riggs. We used a modification (by Dr. Riggs) of the technique for autoradiography of individual DNA molecules which had been developed by Cairns (J. Mol.

Biol. 6, 208 (1963)). Our application of this technique to the DNA of Chinese hamster cells demonstrated the presence in Chinese hamster cell chromosomes of DNA fibers up to 1,800 μ long. Subsequent pulse-labeling studies showed that such long fibers are divided into many shorter replication units, and that DNA replication probably starts in the interior of each unit and then proceeds outward in both directions, at fork-like growing points, to the ends of the unit.

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PART I

THE ISOLATION AND FRACTIONATION OF METAPHASE CHROMOSOMES FROM HELA CELLS

INTRODUCTION

Soon after I had arrived at Caltech, my adviser, Dr. Attardi, suggested the project which eventually resulted in the studies described in this Part. He had conducted preliminary hybridization experiments between DNA and ribosomal RNA from HeLa cells. These experiments had suggested that each HeLa cell contains hundreds of genes for both 28S and 18S ribosomal RNA. The manner in which these hundreds of ribosomal RNA genes are distributed among the 60-70 chromosomes of the HeLa cell was completely unknown at the time, and Dr. Attardi suggested that this would be an interesting question to investigate.

My first approach was to attempt autoradiographic detection of hybridization between ribosomal RNA and whole chromosomes (isolated on a microscopic scale by the method of Prescott and Bender, 1). Unfortunately, this approach proved to be completely unsuccessful.

However, the report of Chorąży, Bendich, Borenfreund & Hutchison (2) on large-scale isolation of metaphase chromosomes from mouse L1210 cells had just appeared, and this suggested an alternative approach to the problem. It should be quite simple, we reasoned, to isolate metaphase chromosomes from HeLa cells by the same method (2), to fractionate them on the basis of sedimentation velocity, and then to test DNA from each of the fractions for capacity to hybridize with ribosomal RNA.

Of course, this approach turned out to be much more complicated than we had anticipated. One major problem was that the method of

Chorąży et al. (2) proved unsatisfactory for isolation of chromosomes from HeLa cells. Therefore, I developed a different method of chromosome isolation which would work for HeLa cells. This method is presented in Chapter I, along with several observations on the properties of the isolated chromosomes.

Additional difficulties were encountered when I tried to fractionate the isolated chromosomes. These difficulties, and the methods used to eliminate or minimize them, are discussed in Chapter II.

The results of the hybridization experiments are also reported in Chapter II. Although some question about the interpretation of the hybridization experiments remains, the results suggest that ribosomal RNA genes are found only in small HeLa cell chromosomes, perhaps only in those chromosomes which carry nucleolar organizers. This conclusion is completely consistent with data from other laboratories (reviewed in Chapter II), obtained after this project was started, which suggest that ribosomal RNA genes are always found exclusively in the nucleolar organizer regions of chromosomes.

CHAPTER I

Isolation of Metaphase Chromosomes from HeLa Cells

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ISOLATION OF METAPHASE CHROMOSOMES FROM HELA CELLS

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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.

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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

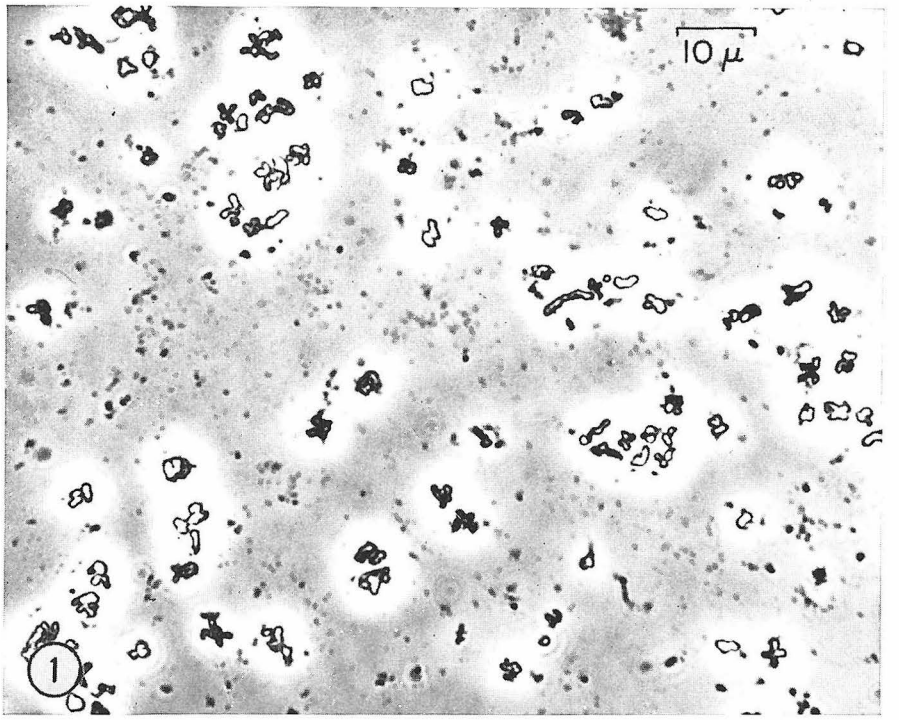

FIGURE 1 Isolated HeLa metaphase chromosomes suspended in HCM. Phase contrast. × 1100.

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

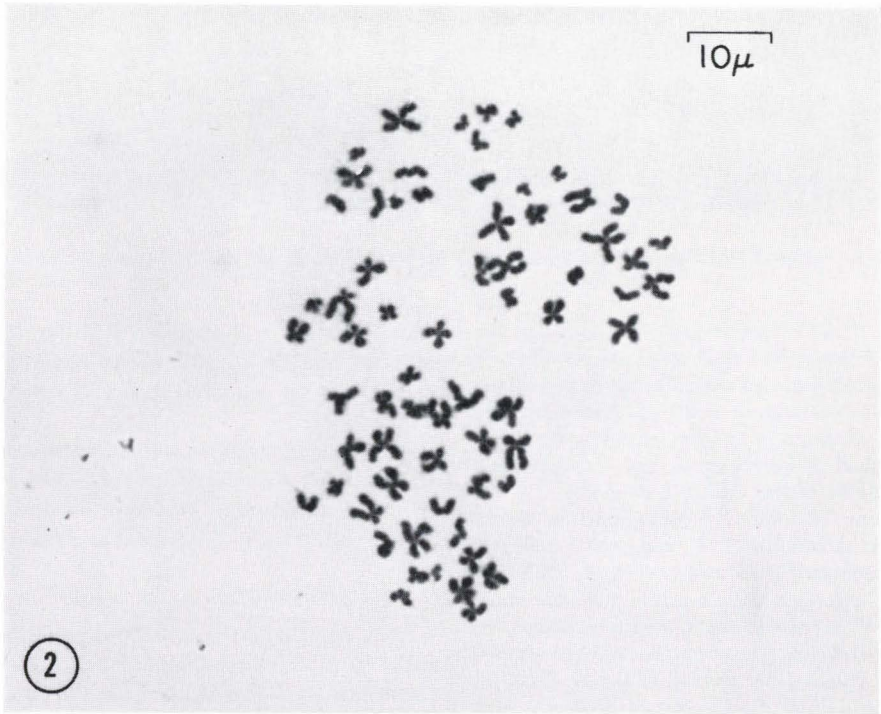

FIGURE 2 The metaphase chromosomes of a single HeLa cell. Bright field. Cells were blocked in metaphase with colchicine, suspended in 1% sodium citrate for 10 min, fixed in acetic acid–ethanol (3:2) for 10 min and then stained in 1% orcein in lactic acid–acetic acid (1:1). Cells suspended in stain solution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

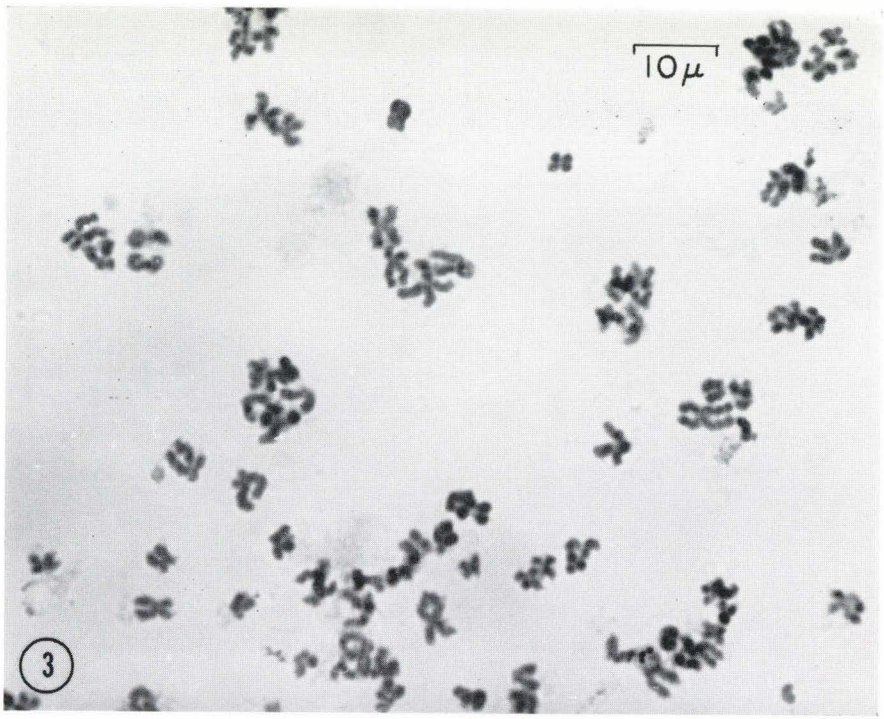

FIGURE 3 Isolated HeLa metaphase chromosomes. Bright field. A small quantity of chromosome suspension in HCM was spread on a glass slide and allowed to dry. The slide was treated with 1% sodium citrate for 10 min, fixed in acetic acid–ethanol (3:2) for 10 min and then stained in 1% orcein in lactic acid–acetic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

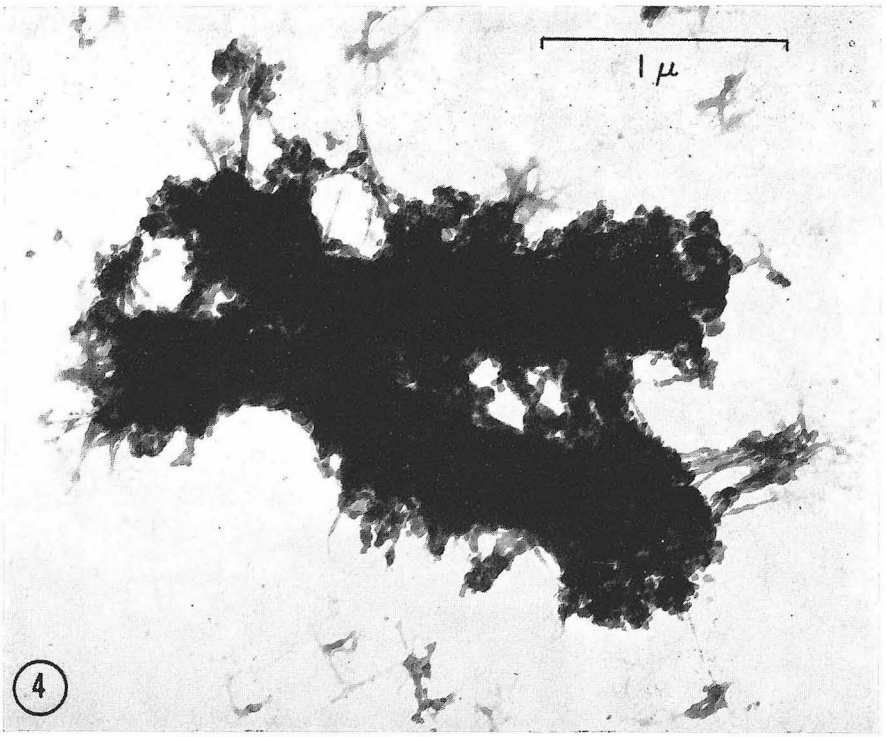

FIGURE 4 Electron micrograph of a typical isolated HeLa metaphase chromosome. Grids were prepared by touching the carbon-Formvar film to the surface of a suspension of chromosomes in HCM, then loading immediately into a grid holder under 30% ethanol. The rest of the procedure has been described by DuPraw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

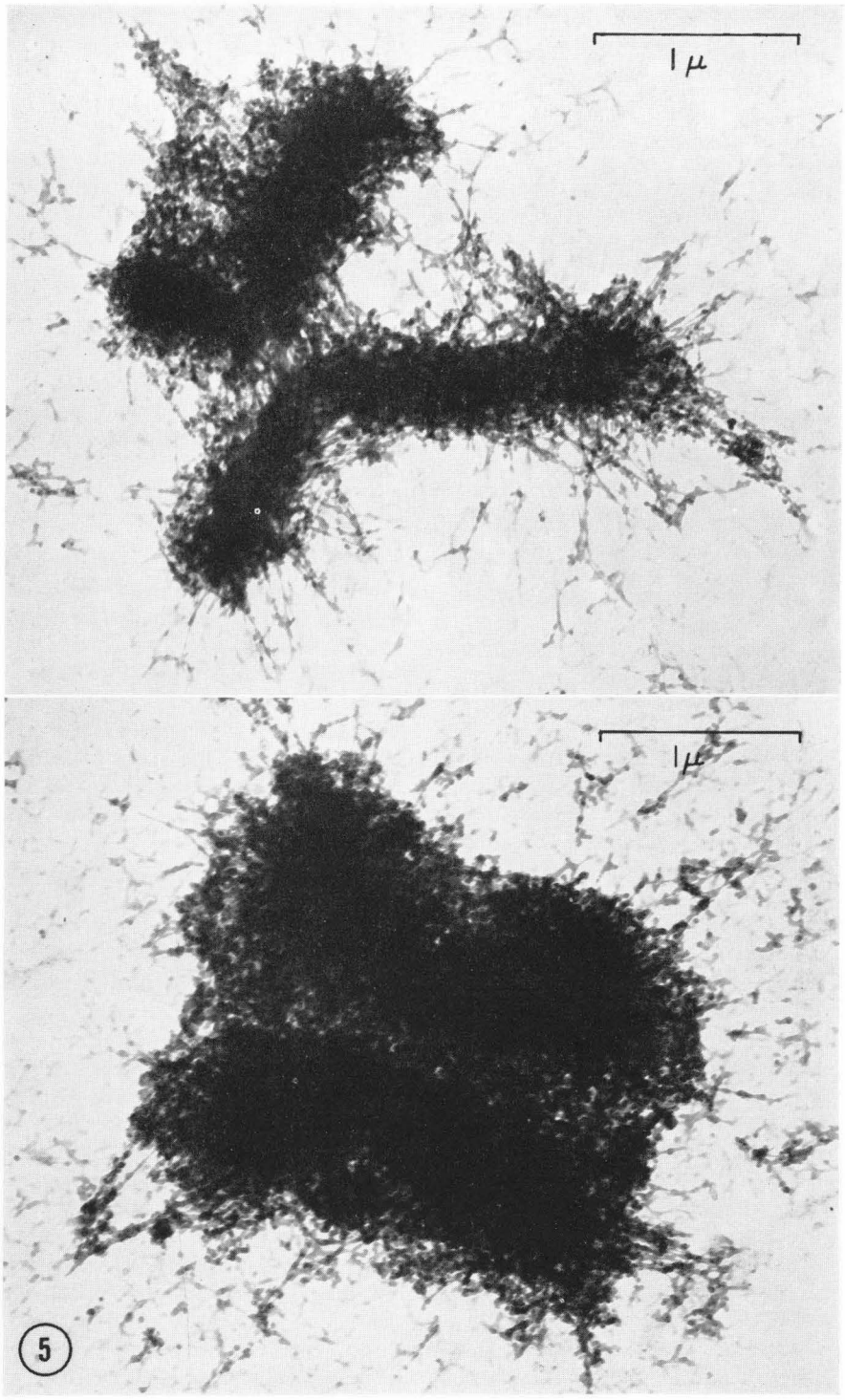

FIGURE 5 Electron micrographs of well preserved, isolated HeLa metaphase chromosomes. Grids were prepared as in Fig. 4. (Courtesy of Dr. E. J. DuPraw.) × 28,000.

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

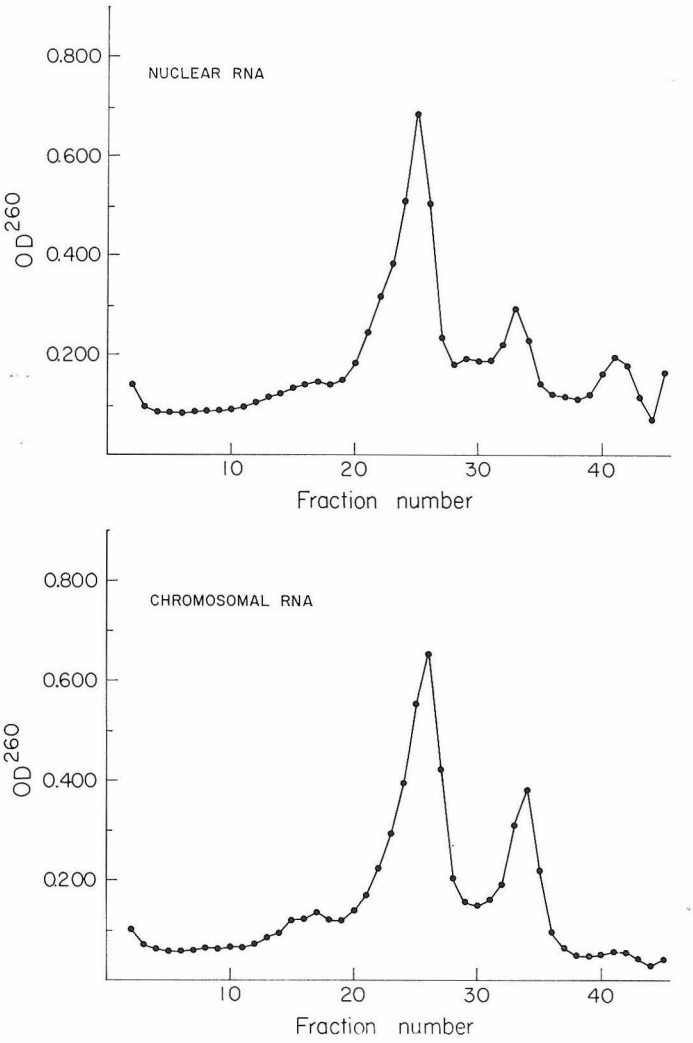

FIGURE 6 RNA was purified (as described in the Materials and Methods section) from a quantity of isolated chromosomes containing about 0.5 mg of DNA and from a quantity of nuclei, isolated as described in Table II, containing about 1.5 mg of DNA. The RNA was dissolved in 0.5 ml of acetate buffer (0.1 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.

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FURTHER DISCUSSION

Since the above report was presented for publication, several new developments have occurred which are relevant to the results contained in the report. First, the method which I have developed for fractionating isolated chromosomes (see Chapter II) has also proved capable of providing considerable further purification. That is, most of the microscopically visible debris which contaminates chromosomes isolated by the method given in this chapter can be removed when such chromosomes are fractionated by the method given in Chapter II. Compare the amounts of debris visible in Figure 3 of Chapter I and in Plate I of Chapter II. Unfortunately, the chemical composition of chromosomes purified by the fractionation procedure has not yet been tested.

Second, the puzzling observation reported in this chapter of unexpectedly large amounts of ribosomal RNA bound to the chromosomes may now be explained. Salzman, Moore & Mendelsohn (3) have recently published yet another method for isolation of chromosomes from HeLa cells. They, too, found that the RNA bound to the chromosomes was mostly ribosomal. In order to determine whether this ribosomal RNA was naturally bound to the metaphase chromosomes in living cells or was bound only after the cells were disrupted for chromosome isolation, they mixed ^{14}C-uridine-labeled HeLa cell ribosomes with unlabeled metaphase HeLa cells and then carried out their usual chromosome isolation. They found that some radioactive ribosomes became bound to the chromosomes. The

quantity of radioactive ribosomes bound suggested that all or nearly all of the ribosomal RNA bound to isolated metaphase chromosomes must be the result of unnatural attachment of cellular ribosomes to the chromosomes during or after cell disruption.

Finally, recent observations by Anil Sadgopal (personal communication) have helped to clarify the nature of the "acid-soluble" protein component of isolated metaphase chromosomes. Note in Table II of this chapter that isolated HeLa cell metaphase chromosomes have a mass ratio of acid-soluble protein: DNA of 2.0 (when HCl is used as acid). This value is nearly twice that observed for interphase chromatin (1.1; see Table II), and it raises the question of whether the histone: DNA ratio is actually higher for metaphase than for interphase chromosomes.

However, Anil Sadgopal has found that if H_2SO_4 is used as acid rather than HCl, the mass ratio of acid-soluble protein: DNA is approximately 1 for both interphase and metaphase chromosomes. Furthermore, by means of acrilamide gel electrophoresis, he has been able to show that while 70% of the proteins extracted from metaphase chromosomes by H_2SO_4 are true histones, only 35% of the HCl-extractable proteins are histones. Thus the histone: DNA ratio in metaphase chromosomes is approximately the same as that in interphase chromatin, and isolated metaphase chromosomes contain a great deal of HCl-soluble non-histone protein not found in interphase chromatin. It is possible that some of this non-histone protein may be ribosomal protein from the ribosomes which are apparently bound to isolated metaphase chromosomes (see above).

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CHAPTER II

Studies of Fractionated HeLa Cell Metaphase Chromosomes I. The Chromosomal Distribution of DNA Complementary to 28S and 18S Ribosomal RNA and to Cytoplasmic Messenger RNA.

by

Joel A. Huberman and Giuseppe Attardi

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SUMMARY

We have developed a method for fractionating isolated HeLa cell metaphase chromosomes on the basis of sedimentation velocity. The method employs low-speed centrifugation of isolated chromosome preparations through glycerol-sucrose density gradients. We have purified DNA from the various chromosome fractions and tested the ability of this DNA to form hybrids with ribosomal RNA and with cytoplasmic messenger RNA. The results show that DNA complementary to ribosomal RNA is confined to the smaller HeLa cell chromosomes (which include those carrying a nucleolar organizer), while DNA complementary to cytoplasmic messenger RNA is distributed among chromosomes of various sizes.

1. INTRODUCTION

One of the principal obstacles to more rapid expansion of our knowledge of mammalian cells has been the difficulty or, in some cases, impossibility of performing genetic experiments, either with whole animals or with cells in tissue culture. Limited classical genetic experiments have been possible with short-lived mammals such as mice. In addition, considerable human genetic information has been obtained from studies of family trees. Another approach to mammalian genetics is offered by the possibility of fusing cells of different genotypes in tissue culture to produce hybrid cells. Recently some progress has been made, by using cell hybridization, in our understanding of genetic regulation in mammalian cells (Harris, Watkins, Ford & Schoefl, 1966; Davidson, Ephrussi & Yamamoto, 1966). In view of the vast amount that remains to be learned about mammalian genetics, however, the successes of classical genetics and of cell hybridization have been quite limited.

We introduce a third experimental approach to mammalian genetics. Although this approach, too, has severe limitations, it is capable of providing information not easily obtainable by the other approaches and therefore should prove a valuable complement to them. This new approach is based on the possibility, recently demonstrated in many laboratories, of isolating intact metaphase chromosomes from mammalian cells. We have fractionated such isolated chromosomes on the basis of their sedimentation velocity and then tested the ability of the DNA purified

from the various chromosome fractions to hybridize with particular kinds of RNA. If one assumes that chromosomes yielding DNA capable of hybridizing with one kind of RNA must contain genes for that RNA, then this approach can provide a crude "mapping" of certain types of genes.

In further discussion we shall use the word "site" to mean a stretch of DNA capable of hybridizing specifically with one molecule of one RNA species.

Previous experiments using hybridization between whole cell DNA and ribosomal RNA have demonstrated a large multiplicity of rRNA[1] sites in eukaryotic cells (for review see Perry, 1967). For example, at least 200 sites have been found for 28S and 18S rRNA in HeLa cells (McConkey & Hopkins, 1964; Attardi, Huang & Kabat, 1965b). The presence of so many rRNA sites in the DNA of each cell raises the question of how these sites are distributed among the chromosomes of the cell.

In the case of both Drosophila melanogaster (Ritossa & Spiegelman, 1965) and Xenopus laevis (Wallace & Birnstiel, 1966) strong evidence now exists for the nucleolar location of rRNA sites. However, there is some question about the location of rRNA sites in HeLa cells. In an earlier attempt to localize 28S rRNA sites in HeLa cells, McConkey & Hopkins (1964) did indeed find a large enrichment of such sites in DNA associated with isolated nucleoli. However, the amount of nucleolus-associated DNA was only large enough to account for about one-seventh of the total 28S rRNA sites in whole cells. The possibility that the remaining 28S rRNA sites were randomly distributed among the chromosomes could not be excluded. The results which we present in this paper

[1]Abbreviations used: rRNA, ribosomal RNA; mRNA, messenger RNA.

demonstrate, however, that all 28S and 18S rRNA sites are clustered on the smaller chromosomes of the HeLa cell and may well be confined entirely to those chromosomes bearing nucleolar organizers.

Our experiments on the chromosomal distribution of sites for pooled cytoplasmic mRNA were undertaken as a control for the results we had obtained with rRNA. We found, as we had anticipated, that mRNA sites are located to about the same extent in all the chromosome fractions.

2. MATERIALS AND METHODS

(a) Chromosome isolation

Chromosomes were isolated by a modification of the method we have described previously (Huberman & Attardi, 1966). HeLa S3 cells growing in suspension culture were exposed for 15 hr to 0.01 ug/ml. of vinblastine sulfate (Maio & Schildkraut, 1966b) and then harvested. By this treatment 50 to 80% of the cells were blocked in metaphase. Further steps were carried out at 0-4°C. The cells were washed twice in buffered isotonic saline (pH 7.4). The pellet of washed cells was gently resuspended in 15 volumes of 0.1 M sucrose, 0.0007M $CaCl_2$, 0.0003 M $MgCl_2$ (Somers, Cole & Hsu, 1963). Five min later, 3 volumes of 0.1 M sucrose, 0.0007 M $CaCl_2$, 0.0003 M $MgCl_2$, 0.0033 M HCl were added slowly, with stirring, to each volume of cell suspension. The final pH was about 3.0. A Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle was then used to homogenize the cells;

homogenization was carried out for sufficient time to break the majority of metaphase cells.

The homogenate was centrifuged at 900 g for 30 min. Each ml. of the resulting pellet, which contained metaphase chromosomes, interphase nuclei, and some cytoplasmic debris, was resuspended in at least 30 ml. of 0.001 M HCl, 0.0007 M $CaCl_2$, 0.0003 M $MgCl_2$, with brief rehomogenization to break up any clumps of chromosomes that might have formed as a result of pelleting. Ten ml. quantities of 2.2 M sucrose in 0.0007 M $CaCl_2$, 0.0003 M $MgCl_2$ (adjusted to pH 3.0 with HCl) were placed in plastic ultracentrifuge tubes, and 15 to 20 ml. quantities of chromosome suspension were layered on top. The upper three-fourths of the tube contents were then gently stirred to form a rough density gradient. The tubes were centrifuged at 80,000 g for 1 hr. Nearly all chromosomes and nuclei sedimented to the bottom, while most cytoplasmic debris floated above the 2.2 M sucrose layer.

(b) Chromosome fractionation

The pellets of chromosomes and nuclei were resuspended in a small volume of 0.001 M HCl, 0.0007 M $CaCl_2$, 0.0003 M $MgCl_2$ (about 5 ml. per pellet). One-ninth volume of 0.2 M tris, pH 7.4, 0.01 M $CaCl_2$, 0.5% saponin was added to the suspension and mixed. The suspension was then further diluted with at least one volume of 0.02 M tris, pH 7.0, 0.002 M $CaCl_2$, 0.05% saponin (FM or fractionation medium) and centrifuged at 900 g for 30 min.

Each ml. of the resulting pellet was resuspended in at least 10 ml. of FM and homogenized briefly by hand with a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N.J.) to break up clumps. Use of a Potter-Elvehjem homogenizer rather than a Dounce homogenizer may result in morphological damage to some of the chromosomes. To each 10 ml. of suspension 3.3 ml. of glycerol were then added and mixed thoroughly.

Ten to fifteen ml. of suspension were gently layered onto 140 ml. of a linear gradient from 30% (w/w) sucrose in FM (on the bottom) to 30% (w/w) glycerol in FM (on the top) which had been formed in a 250 ml. glass centrifuge bottle. The gradient was centrifuged at 450 g in a swinging bucket type rotor for 40 min (4°C) with careful acceleration and deceleration to prevent mixing. Fractions of a convenient size (usually 4 ml.) were collected through a thin glass tube inserted to the bottom of the centrifuge bottle.

In most experiments the fractions were pooled into classes containing approximately equal amounts of material and the chromosomes from each class were collected by centrifugation at 50,000 g for 10 min.

(c) Nucleic acid preparations

DNA was prepared from whole HeLa cells, chromosomes, or other cell fractions by the Marmur procedure (Marmur, 1961).

Ribosomal RNA labeled to a high specific activity with ^{32}P was prepared as described elsewhere (Attardi, Huang & Kabat, 1965b).

Cytoplasmic mRNA was prepared from the polysome fraction of HeLa cells exposed to ^{3}H - uridine (1.25 μC/ml., 17 mC/μM) for 45 min. The method for polysome isolation has been previously described (Houssais & Attardi, 1966) as has the method for RNA extraction from polysomes (Attardi, Parnas, Hwang & Attardi, 1966).

(d) Hybridization procedures

DNA and rRNA were hybridized under conditions described previously (Attardi, Huang & Kabat, 1965a, b) with the following modifications: Mixtures containing heat-denatured DNA (20 μg/ml.) and ^{32}P-labeled 28S or 18S rRNA (1 μg/ml.) in 2X SSC (0.3 M NaCl, 0.03 M Na citrate) were incubated at 70°C for 2.5 hr, then cooled slowly to room temperature. After ribonuclease treatment and passage through a column of Sephadex G-100, the hybridized material was collected on membrane filters and washed at 60°C. In some experiments the hybridized RNA was eluted from the filters and then centrifuged through a sucrose gradient at 24,500 rev./min (SW-25.1 rotor, Spinco Model L centrifuge) for 24 to 27 hr in the case of 28S RNA or for 38 hr in the case of 18S RNA.

Cytoplasmic mRNA was hybridized with DNA under conditions identical to those for rRNA, except that the incubations were performed with 8 μg/ml. RNA and 20 μg/ml. DNA, and that filters were washed at 55°C.

3. RESULTS

(a) Chromosome fractionation

(i) The method.

Although preliminary reports of metaphase chromosome fractionation have appeared previously (Maio & Schildkraut, 1966a; Huberman & Attardi, 1966), this is the first detailed account of the procedures involved and the results obtained. The reader may be interested, therefore, in a brief description of the advantages and drawbacks of the fractionation method we have chosen — sedimentation velocity fractionation in a density gradient.

The overwhelming advantage of sedimentation velocity fractionation is that it works. None of the other techniques we have tried so far has produced a convincing and practical chromosome fractionation. A list of these techniques follows:

1. Equilibrium centrifugation in a buoyant sucrose density gradient. In our experiments, which were all carried out at pH 3, the vast majority of chromosomes formed a thin band at a density of about 1.31 g/ml. A few chromosomes were found, for unknown reasons, at positions of slightly higher density. Small numbers of chromosomes were also found at positions of lower density, ranging from the chromosome band to the regions of cytoplasmic debris. In most cases pieces of cytoplasmic debris were obviously attached to these "light" chromosomes. There was no obvious selection of any one morphological type of chromosome at any position in the gradients.

2. Electrophoresis in a sucrose density gradient. We used the apparatus of Olivera, Baine & Davidson (1964), and formate or acetate buffers at an ionic strength of 0.01 (Perrin, 1963). No differences in mobility could be demonstrated for chromosomes of different morphological types. Buffers ranging from pH 3.2 to pH 5.2 were tried. The isoelectric pH of the chromosomes was measured to be about 4.6.

3. Filtration. Selective filtration experiments were attempted with Millipore filters (Millipore Corp., Bedford, Mass.) and Nuclepore filters (General Electric Co., Pleasanton, Calif.) of pore sizes ranging from about 2 to 10 μ. Although both filter types prevented the passage of most chromosomes larger than their pore sizes, both also became rapidly clogged.

Although sedimentation velocity fractionation proved, even in our initial experiments, to be much more successful than the techniques listed above, we soon learned that its resolution is limited by several factors. First, the differences in sedimentation velocity between the various morphological groups of HeLa cell chromosomes have turned out to be small, while variations in the state of contraction of the isolated chromosomes have proved undesirably large. These variations in contraction produce sufficient spread in the sedimentation velocity of the various chromosome groups to effectively prevent complete resolution of groups of similar size.

However, the most serious limitation is the tendency of the chromosomes to aggregate and form clusters. We have encountered two types of chromosome clustering. First, some of the chromosome sets present

in metaphase cells before homogenization are dispersed incompletely or not at all during homogenization, leaving clusters of two or more chromosomes. The chromosomes in these clusters are bound to each other in some manner that is very resistant to further homogenization. We have found no effective way to remove clusters of this type; fortunately only a small proportion of chromosomes is involved.

The second type of clustering, which can be much more frequent, is the result of aggregation occurring after homogenization. Some clusters of this type are always formed by pelleting. In addition such aggregation can occur spontaneously in suspension. Although clusters of this type can easily be redispersed by mild homogenization, they present a problem in sedimentation velocity fractionations because they can form during the process of sedimentation itself.

Although we have not been able to eliminate the problem of such clustering completely, we have been able to minimize it in two ways. First, we use low chromosome concentrations during fractionation to reduce the frequency of chance contacts. Good results are obtained when the chromosome concentration in the suspension layered over the glycerol-sucrose gradients is 10^9 per ml. or less. Second, we use a medium in which the probability of aggregation as a result of chance contact is minimized. For this purpose we have found that FM (0.02 M tris, pH 7.0, 0.002 M $CaCl_2$, 0.05% saponin), a modification of a medium for chromosome studies suggested by Maio and Schildkraut (1966b), gives best results. The saponin in this medium helps to reduce the probability of chromosome aggregation. Since divalent cations tend to increase the

probability of chromosome aggregation, the concentration of $CaCl_2$ was reduced to 0.002 M, which is the minimum necessary to maintain satisfactory chromosome contraction at pH 7.

We have also attempted to minimize the limitation on resolution due to the small differences in sedimentation velocity between the various chromosome groups. In conventional sucrose gradients, a strong viscosity gradient is also present. Since the viscosity increases with distance from center of rotation, the sedimentation of large particles is impeded more than that of small particles and the final separation between large and small particles is reduced. We have considerably decreased this viscosity gradient, while maintaining a good density gradient, by using 30% glycerol ($\rho^{20°/4°}$ = 1.0727 g/ml., $\eta^{20°}$ = 2.50 cp) rather than water ($\rho^{20°/4°}$ = 0.9982 g/ml., $\eta^{20°}$ = 1.00 cp) or sucrose of a comparable density (18%; $\rho^{20°/4°}$ = 1.0721 g/ml., $\eta^{20°}$ = 1.79 cp) as light solution in a density gradient, with 30% sucrose ($\rho^{20°/4°}$ = 1.1270 g/ml., $\eta^{20°}$ = 3.19 cp) as heavy solution. We found considerable improvement in the separation of chromosome groups with such glycerol-sucrose gradients. In addition we have found that the increased overall viscosity of such gradients greatly improves their stability against convection.

Another problem of the sedimentation velocity fractionation method should be mentioned: there is a selective loss of large chromosomes. Part of this loss results from selective impact of large chromosomes against the walls of the centrifuge bottle. Since sedimentation is radial while the centrifuge bottles have a cylindrical shape, the

probability that a chromosome will hit a wall of the bottle is greater for chromosomes which tend to move further down the bottle. The rest of the large chromosome loss is due to aggregation. Large chromosomes aggregated with other chromosomes of any size sediment so rapidly that they are usually lost to the bottom of the gradient while small chromosomes are usually lost only if they have aggregated with large chromosomes.

(ii) Description of fractionation

The results of a typical fractionation, in terms of chromosome distribution, are shown in Fig. 1. Notice that different chromosome types are not resolved into discrete peaks. Instead, a rather smooth distribution is obtained. In all the experiments described here, this smooth distribution was arbitrarily divided into four parts. A typical division is shown in Fig. 1. Here chromosome class (1) was intended to include the largest chromosomes while class (4) was intended to include the smallest chromosomes. The chromosomes found below class (1) were mostly in clusters. The chromosomes found above class (4) were predominantly ones removed accidentally from the bottom and sides of the centrifuge bottle during collection of fractions.

Rather large quantities of DNA were required from each chromosome class for the hybridization experiments described in this article. To provide sufficient DNA, the corresponding chromosome classes from six to nine separate fractionations were pooled for DNA extraction. No appreciable loss of resolution resulted from pooling. Plate 1 shows photographs of the pooled chromosome classes which provided DNA for the first set of hybridization experiments (chromosome preparation I).

Figure 1. Distribution of chromosomes after centrifugation through a glycerol-sucrose density gradient.

Chromosomes were isolated from about 3×10^8 HeLa cells and suspended in 10 ml. of FM plus 3.3 ml. of glycerol as described in Materials and Methods. The chromosome suspension was layered onto 140 ml. of a linear gradient from 30% glycerol to 30% sucrose in FM and centrifuged 40 min at 450 g ($4^{\circ}C$). Fractions were collected as described in Materials and Methods. The chromosome concentration in even-numbered fractions was determined by counting in a bacterial counting chamber. Standard deviations are indicated by the error bars.

The cut-off points for pooling of the fractions into separate chromosome classes are indicated by arrows.

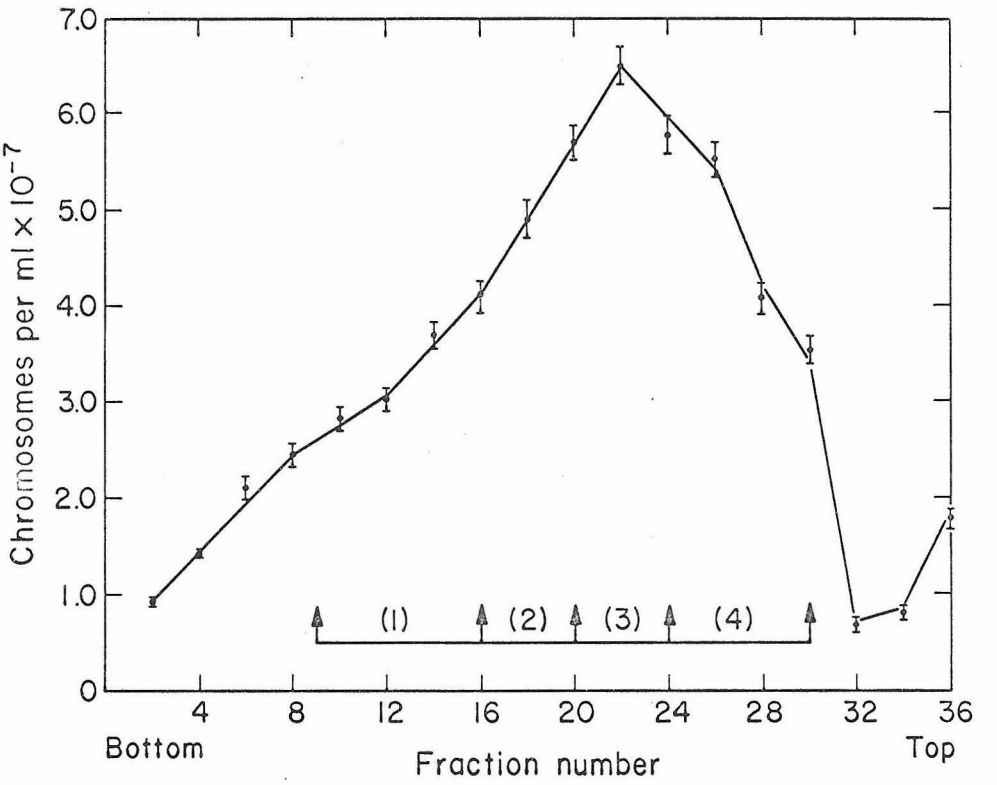
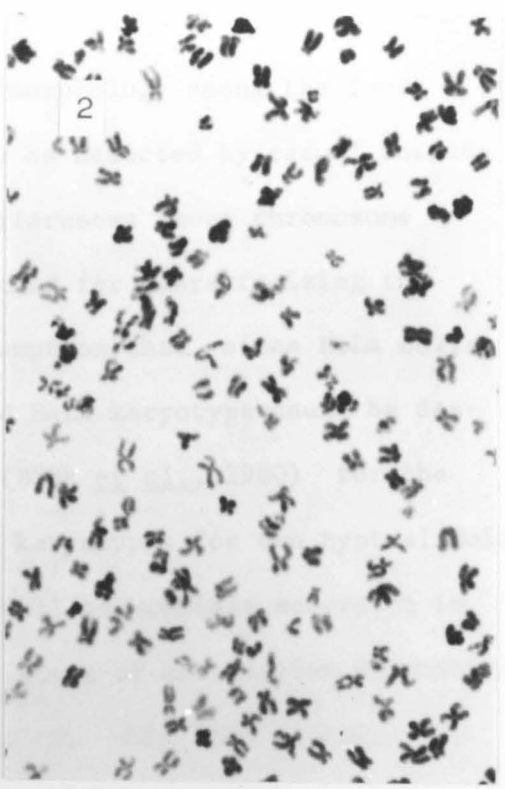

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
Chromosomes per ml x 10⁻⁷
(1)
(2)
(3)
(4)
4
8
12
16
20
24
28
32
36
Bottom
Fraction number
Top

Figure 1.

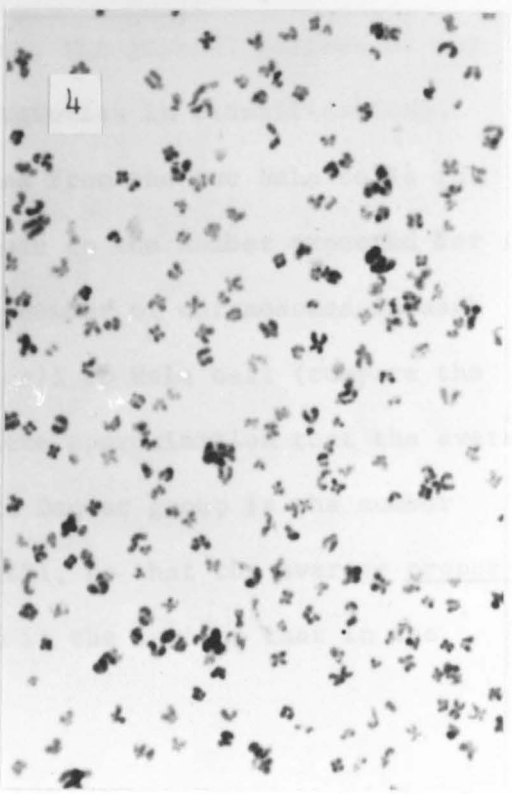
Plate I. Isolated and fractionated HeLa metaphase chromosomes.

A small amount of a suspension in FM containing 30% glycerol of each pooled chromosome class from chromosome preparation I (see text) was spread on a clean glass slide and allowed to dry for 60 min. The half-wet chromosomes were then fixed in acetic acid-ethanol (3:2 by vol) for 10 min and stained in 1% orcein in lactic acid-acetic acid (1:1 by vol). Bright field (x 900).


1


2


3

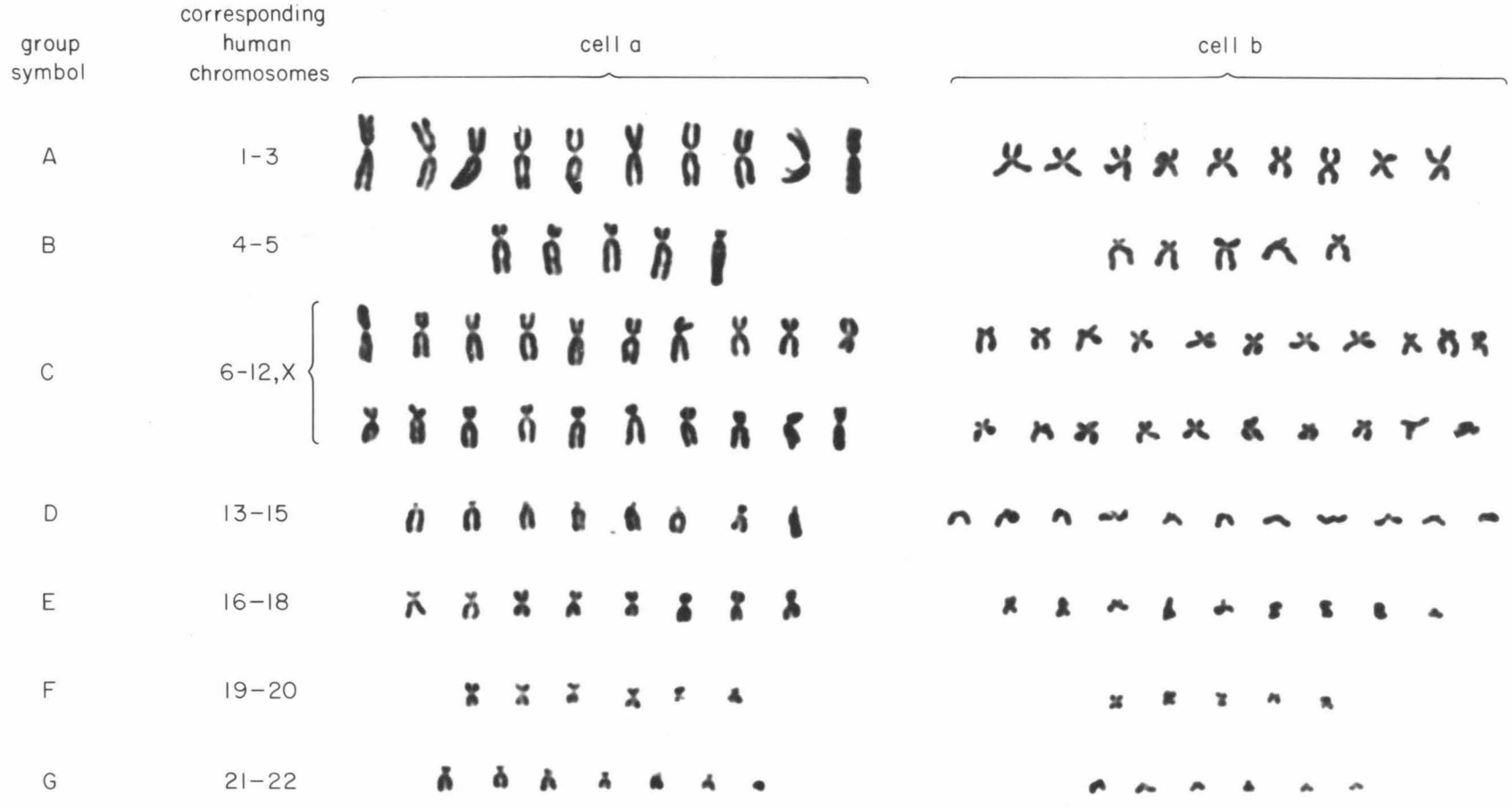

4

Although differences in chromosome morphology among the four chromosome classes in Plate I can easily be detected by casual inspection, quantitative evaluation of the differences among chromosome classes was not a trivial task. Our method for characterizing the chromosome classes was based on the assumption that, since HeLa cells are of human female origin, the aneuploid HeLa karyotype could be described in terms of the "Denver system" (Böök et al., 1960) for the human karyotype. In Plate II are shown karyotypes for two typical HeLa cells with the chromosomes arranged as well as possible according to the "Denver system". The difference in state of contraction between the two chromosome sets will be discussed below. Note that although the identification of a few chromosomes in the karyotypes shown may be dubious, most chromosomes can be placed unambiguously in one of the Denver groups (as will be clear later on, the general pattern of our results is not affected by minor uncertainties in classification). Note also that the number of chromosomes from the two HeLa cells in each of the Denver groups is fairly close to the number expected for a triploid human female cell. Since the number of chromosomes in each of the Denver groups varies from HeLa cell to HeLa cell (compare the two cells shown in Plate II), we made the approximation that the average number of HeLa cell chromosomes in each Denver group is the number expected for a triploid human female cell, so that the average proportion of HeLa chromosomes in each group is the same as that in the human female diploid complement.

Plate II. Karyotypes of two individual HeLa cells

Cells were blocked in metaphase with colchicine (5×10^{-6} M), suspended in 1% sodium citrate for 10 min to allow spreading of chromosomes, fixed in acetic acid-ethanol (3:2 by vol) for 10 min and then stained with 1% orcein in lactic acid-acetic acid (1:1 by vol). Cells suspended in stain solution were squashed by thumb pressure between a slide and a cover slip. Photographic enlargements were made of the metaphase chromosome sets of single cells. The images of individual chromosomes were cut out of the enlargements, arranged as well as possible into morphological groups according to the Denver system (Böök et al., 1960), and rephotographed. Bright field (x 1300).

"Group symbols" are the letter symbols commonly applied to the morphological groups of the Denver system. The numbers assigned by the Denver system to the human chromosomes in different groups are given for comparison.


group symbol
corresponding human chromosomes
cell a
cell b
A
1-3
B
4-5
C
6-12,X
D
13-15
E
16-18
F
19-20
G
21-22

From photographs like those in Plate I we attempted to determine the proportion of chromosomes from the various Denver groups in each of the fractionated chromosome classes. A number of difficulties were encountered:

1. Variations in the state of contraction of chromosomes from different cells produced a completely continuous spectrum of chromosome sizes. An example of such variations is given by the two cells in Plate II. These variations are probably the result of differences between cells in the duration of metaphase arrest. As a consequence of such variations, arbitrary judgments frequently had to be made between Denver groups of similar morphology but different size (between groups A and C, for example). Some feeling for the problem can be gained by imagining an attempt to classify chromosomes in a mixture of the two karyotypes shown in Plate II.

2. Occasionally chromosomes in the photographs were found in tight clusters. In these cases it was difficult or impossible to determine the morphology of the chromosomes. Such chromosomes were excluded from the sample.

3. Morphological comparison of the fractionated chromosomes with the chromosomes (like those in Plate II) from whole metaphase cells shows that a few chromosomes were distorted slightly in drying on the slides (or at some other step in the whole procedure) so that the relative lengths of the chromosome arms were altered. A continuous spectrum of centromere positions, ranging from metacentric to apparent telocentric, was produced. Thus arbitrary judgments occasionally had

to be made between Denver groups of similar size but different morphology (between groups D and E, for example).

Despite the above-listed difficulties we feel that this method of characterization is sufficiently reliable to provide a valid basis for analysis of the results of hybridization experiments. We have considered alternative characterization methods such as simple chromosome length measurement, but in our opinion characterization based on the "Denver system" is at least as "accurate" as other forms of characterization and certainly more meaningful.

Since we intended to compare the results of characterization of the fractionated chromosome classes with the results of hybridization experiments, we decided to express the characterization results in terms of the enrichment of DNA from chromosomes of each Denver group in each fractionated chromosome class. Our method of calculation follows:

1. For each fractionated chromosome class, (n), we determined from a randomly chosen series of photographs the number, P, of chromosomes in each Denver group, X. In this process we pooled chromosomes of groups E and F in view of the difficulty in distinguishing them.

2. We multiplied the number of chromosomes in each Denver group, $P_{X,n}$ by the amount of DNA contained in an average human metaphase chromosome of that group, D_X (see Table 1).

3. Using the products from the above step, we calculated the fraction of chromosome class DNA due to chromosomes of each Denver group.

TABLE 1

Approximate Amounts of DNA Contained in Human Metaphase Chromosomes

X	$P_{X,h}$	D_X	$P_{X,h}\ D_X$
Denver group	Number chromosomes in group (diploid female)	Amount of DNA per average metaphase chromosome* (μμg)	Amount of DNA per group at metaphase* (μμg)
A	6	0.59	3.52
B	4	0.49	1.97
C	16	0.40	6.36
D	6	0.27	1.61
EF	10	0.20	2.03
G	4	0.13	0.51

*These values are calculated from the data of Mendelsohn et al. (1966) who estimated the relative DNA content of individual human female chromosomes photometrically, and from the approximate value of 8 μμg DNA for the basal DNA content of a human diploid cell (Leslie, 1955).

The symbols X, $P_{X,h}$ and D_X are defined in the text.

4. We divided the fraction of chromosome class DNA due to each Denver group by the fraction of total DNA due to the same Denver group in a human female metaphase cell (see Table 1) to obtain the "enrichment factor" for that group.

Thus the enrichment factor for any Denver group X, in any chromosome class (n), is given by

$$\text{Enrichment factor } (X,n) = \frac{\text{Fraction of group (n) DNA due to group X chromosomes}}{\text{Fraction of total cell DNA due to group X chromosomes}}$$

$$= \frac{P_{X,n}\, D_X}{\sum_{X=A}^{G} (P_{X,n}\, D_X)} \Big/ \frac{P_{X,h}\, D_X}{\sum_{X=A}^{G} (P_{X,h}\, D_X)}$$

where $P_{X,h}$ is the number of chromosomes of group X in a human female diploid cell (see Table 1). According to our approximation that the average number of chromosomes in a Denver group in HeLa cells is the same as the number expected for a triploid human female cell, the denominator of the enrichment factor could apply to total HeLa cell DNA as well as to total human cell DNA.

Enrichment factors for the two fractionated chromosome preparations used in hybridization experiments are presented in Table 2. Note that while classes (3) and (4) are fairly free of large chromosomes, classes (1) and (2) are contaminated to a considerable extent by small chromosomes. This is a consequence of chromosome clustering; clusters of small chromosomes sediment like larger chromosomes and thus contaminate the classes containing large chromosomes.

TABLE 2

Enrichment Factors for the Chromosome Preparations Used in Hybridization Experiments

Chromosome preparation	Class number	Denver Group					
		A	B	C	D	EF	G
I	(1)	1.3	1.2	1.2	0.6	0.5	0.7
	(2)	0.3	0.5	1.6	0.8	0.8	0.7
	(3)	0.1	0.2	1.3	1.5	1.2	1.0
	(4)	0.1	0.2	0.2	2.2	2.3	4.3
II	(1)	1.1	1.1	1.0	0.9	0.8	1.1
	(2)	0.9	1.1	1.3	0.8	0.5	0.7
	(3)	0.1	0.4	1.3	1.8	1.5	1.4
	(4)	0.2	0.2	0.5	2.2	3.1	4.0

Enrichment factors, class numbers, and the method used to obtain the data are defined in the text.

(iii) Recentrifugation of chromosome classes

We have found that some improvement in resolution can be obtained by recentrifuging chromosome classes through glycerol-sucrose gradients. The enrichment factors for a preparation in which each class was recentrifuged once are given in Table 3. Note that while recentrifugation gives a negligible change in the contamination of classes (1) and (2) by small chromosomes, it eliminates contamination of classes (3) and (4) by large chromosomes. However, the overall improvement obtained by recentrifugation was not considered worth the added effort and attendant chromosome loss for the large-scale preparations used in hybridization experiments.

(b) Hybridization with 28S and 18S rRNA

(i) Results with chromosome preparations

All hybridization experiments with rRNA were performed at an RNA:DNA ratio of 1:20. Under the conditions used in the present experiments, this ratio is sufficient to saturate the 28S or 18S rRNA sites of HeLa cell DNA (Attardi, Huang & Kabat, 1965b).. The quantity of RNA specifically hybridized was usually determined from sedimentation profiles of that RNA which was eluted from membrane filters (Attardi, Huang & Kabat, 1965a, b). An example of such sedimentation profiles, obtained after hybridization of 28S rRNA with DNA from chromosome preparation I, is shown in Fig. 2. Also shown is the sedimentation profile of 28S rRNA (input RNA) which was subjected to the same thermal treatment as the hybridized RNA. Note that the hybridized RNA samples sediment

TABLE 3

Enrichment Factors for Recentrifuged Chromosome Classes

Class number	Denver Group					
	A	B	C	D	EF	G
(1)	1.4	1.3	0.9	0.7	0.6	0.7
(2)	0.3	0.4	1.5	1.4	1.0	0.9
(3)	< 0.1	<0.1	0.6	3.1	3.0	2.0
(4)	<0.1	<0.1	<0.1	1.9	4.5	6.5

Chromosome classes prepared as described in Materials and Methods were each resuspended in FM-glycerol and layered a second time over standard glycerol-sucrose gradients. Recentrifugation was performed in the standard manner. For each class only those fractions corresponding to that class in the first centrifugation (e.g. see Fig. 1) were pooled.

Enrichment factors, class numbers, and the method used to obtain the data are defined in the text.

Figure 2. Sedimentation profiles of 28S rRNA hybridized with chromosomal and total HeLa cell DNA.

Hybridization between ^{32}P-28S rRNA and DNA from the sources indicated (whole HeLa cells and chromosome classes (1) through (4) of chromosome preparation I) was performed as described in Materials and Methods. The hybridized RNA was eluted from membrane filters as described in Materials and Methods and run on 5-20% linear sucrose gradients in 0.02 M potassium phosphate buffer, pH 7.4, 0.1 M NaCl, containing 1% CH_2O, for 24 hr at 24,500 rev./min in the Spinco SW 25.1 rotor. Fractions were collected and analyzed for acid-insoluble radioactivity. The radioactivity measurements were converted to amounts of RNA per ml. divided by the total amount of corresponding DNA retained on the membrane filter (left scale).

The "input 28S rRNA" was subjected to the same medium conditions and thermal treatments used for hybridization and elution of RNA from filters, then run in a sucrose gradient as described above. Fractions were collected and acid-insoluble radioactivity was determined (right scale).

The vertical dashed line indicates the position of the mode of the input 28S rRNA profile.

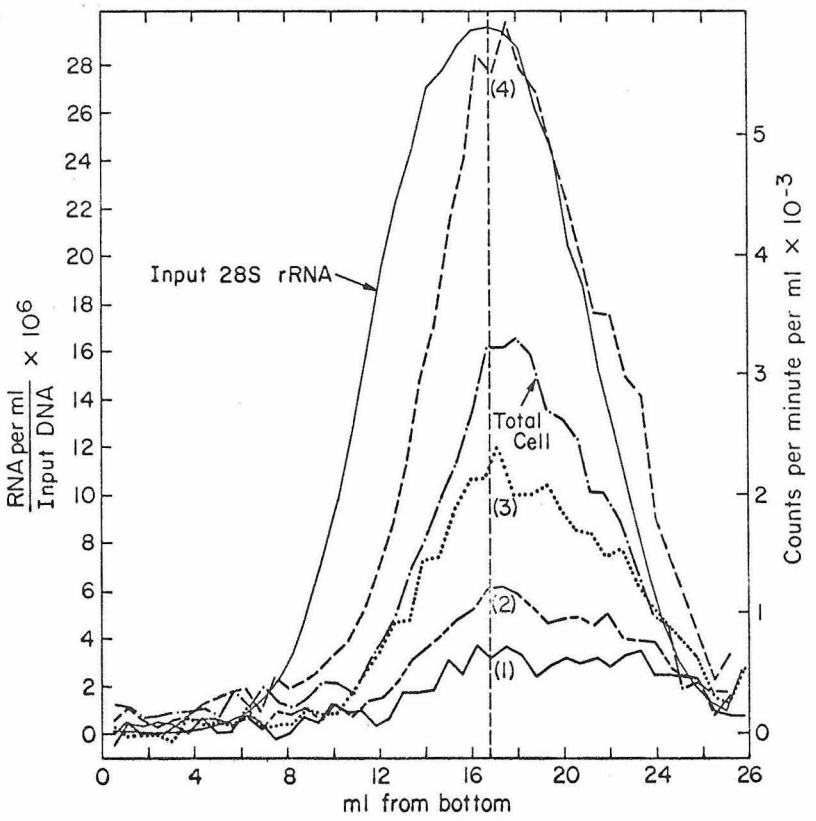

Input 28S rRNA
(4)
Total Cell
(3)
(2)
(1)
RNA per ml / Input DNA × 10^6
Counts per minute per ml × 10^-3
ml from bottom
0
4
8
12
16
20
24
26
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
0
1
2
3
4
5

Figure 2.

somewhat more slowly than the input RNA. Since RNA hybridized with complete specificity ought to sediment as rapidly as input RNA, the quantity of RNA specifically hybridized was estimated from that portion of the sedimentation profile to the left of the mode of the input RNA profile. In practice, the modal position of the input RNA profile was determined (dashed line in Fig. 2), the quantity of hybridized RNA to the left of the modal position was measured, and this quantity was multiplied by 2 to account for symmetrical distribution about the modal position.

In some experiments (indicated in tables) sedimentation analysis of the hybridized RNA was not performed. Instead the total quantities of RNA retained on the membrane filters were determined. Relative values obtained in this way proved to be similar to the relative values obtained by sedimentation analysis.

Hybridization results, whether determined by sedimentation analysis or by measurement of total RNA retained on filters, are expressed throughout this paper as a ratio to the amount of hybrid obtained with whole HeLa cell DNA under identical conditions. Thus

$$\text{Hybrid ratio} = \frac{\text{Amount of hybrid obtained with DNA being analyzed}}{\text{Amount of hybrid obtained with whole HeLa cell DNA}}$$

Notice that hybrid ratios are so calculated that, in the special case when a particular kind of RNA site is confined to one Denver group, the hybrid ratios for that kind of RNA will equal the corresponding enrichment factors for that Denver group. Absolute hybrid values can be determined from the hybrid ratios and from the absolute hybrid values

for whole HeLa cell DNA. We have found that a fraction equal to 2.1×10^{-4} of HeLa cell DNA hybridizes with 28S rRNA and that 1.5×10^{-4} of HeLa cell DNA hybridizes with 18S rRNA as measured by the amount of RNA retained on membrane filters. When the hybrid values are determined by sedimentation analysis, the corresponding figures are 1.2×10^{-4} for 28S rRNA and 0.9×10^{-4} for 18S rRNA. The latter two values are somewhat higher than values determined previously by the same method (Attardi, Huang & Kabat, 1965b). The difference is due to the shorter centrifugation times used in the present experiments which resulted in increased overlap between the sedimentation profiles of input RNA and hybridized RNA.

Hybrid ratios for hybridization between the two species of rRNA and DNA from chromosome preparations I and II are shown in Table 4. Notice that the hybrid ratios for both species of rRNA are lowest for chromosome classes (1) and (2) and highest for class (4) (the class enriched in the smaller chromosomes). In contrast to this trend for the hybrid ratios, the enrichment factors for Denver groups A, B and C (see Table 2) are lowest in chromosome classes (3) and (4). Thus rRNA sites cannot be significantly localized on chromosomes of groups A, B, or C.

On the other hand, the enrichment factors for groups D, EF, and G all increase with chromosome class number, having maxima in class (4). Therefore a more discriminating kind of analysis is required to determine which of these groups contain rRNA sites. As pointed out above, if any one group were to contain all the rRNA sites, the enrichment factors for that group would be equal to the corresponding hybrid ratios for

TABLE 4

Results of Hybridization between rRNA and Chromosomal DNA

Chromosome preparation	Class number	Hybrid ratio with 28S RNA	Hybrid ratio with 18S RNA	Enrichment factor for group $\frac{3D + 2G}{5}$
I	(1)	0.2*	0.4*	0.6
	(2)	0.4*	0.3*	0.8
	(3)	0.7*	0.8*	1.3
	(4)	1.8*	2.6*	3.0
II	(1)	0.6	-	1.0
	(2)	0.5	-	0.8
	(3)	0.7	-	1.6
	(4)	1.8	-	2.9

*Hybrid ratio checked by sedimentation analysis of hybridized RNA.

Class numbers, hybrid ratios, and enrichment factors are defined in the text. Hybridization experiments were performed as described in Materials and Methods.

rRNA. If more than one group were to contain the rRNA sites then weighted averages (taking into account the different numbers of rRNA sites in each group) of the enrichment factors for the responsible groups would also be equal to the corresponding hybrid ratios. Such weighted averages of enrichment factors for a combination of groups D and G are shown in Table 4. Calculation of these averages was based on the assumption that, on the average, each G chromosome would contain the same number of rRNA sites as each D chromosome; therefore, the enrichment factors for group D were weighted by 3 and the enrichment factors for group G were weighted by 2 to account for the fact that the human haploid karyotype contains three D chromosomes and two G chromosomes. We have chosen this particular combination of groups for discussion because D and G chromosomes are thought to be the chromosomes which carry nucleolar organizers in human cells (Ohno, Trujillo, Kaplan & Kinosita, 1961; Ferguson-Smith & Handmaker, 1963; Yerganian, 1963; Ferguson-Smith, 1964).

Careful comparison of the enrichment factors for groups D, EF, and G (Table 2) with the rRNA hybrid ratios (Table 4) shows that in nearly every case the enrichment factors are considerably greater than the corresponding hybrid ratios. It is therefore impossible to account for all the rRNA sites of whole HeLa cell DNA with these chromosome groups. Since we have already excluded chromosome groups A, B, and C from containing a detectable number of rRNA sites, some rRNA sites apparently are unaccounted for. This discrepancy could arise from a reduction of hybridization capacity suffered by the chromosomal

DNA during the isolation and fractionation procedures. We shall examine this loss of hybridization capacity in detail below and in the Discussion section.

If hybridization capacity is reduced to the same extent for DNA from all chromosome classes, then, for the chromosome group or combination of groups containing all the rRNA sites, the quantity

$$\frac{\text{hybrid ratio}}{\text{enrichment factor}}$$

should be constant for all chromosome classes. In Figure 3 this quantity is plotted against chromosome class number for groups D, EF, G and $\frac{3D + 2G}{5}$.

Notice in Fig. 3a that, in the case of 28S rRNA, the combination group $\frac{3D + 2G}{5}$ appears to give the closest approach to constancy. In the case of 18S rRNA (Fig. 3b), both group G and the combination $\frac{3D + 2G}{5}$ seem to give more constant values than groups D or EF. This better correlation between the hybrid ratios and group $\frac{3D + 2G}{5}$ is consistent with exclusive confinement of rRNA sites to the D and G chromosomes (the chromosomes bearing nucleolar organizers). If this is true, then the magnitude of the deviations from constancy shown by group $\frac{3D + 2G}{5}$ in Fig. 3 is an indication of the magnitude of deviations to be expected as a result of experimental error. In Fig. 3a the difference in values shown by group $\frac{3D + 2G}{5}$ for chromosome preparations I and II is also an indication of experimental error. Differences between chromosome preparations shown by the other groups are not necessarily due to experimental error, however. Instead such differences would be anticipated as a result of fluctuations between preparations in the relative porportions of group

Figure 3. Correlation between hybrid ratios and enrichment factors for hybridization between DNA of chromosome classes (1) through (4) and (a) 28S or (b) 18S rRNA.

Values are calculated from the data in Tables 2 and 4. o——o, DNA from chromosome preparation I; Δ——Δ , DNA from chromosome preparation II.

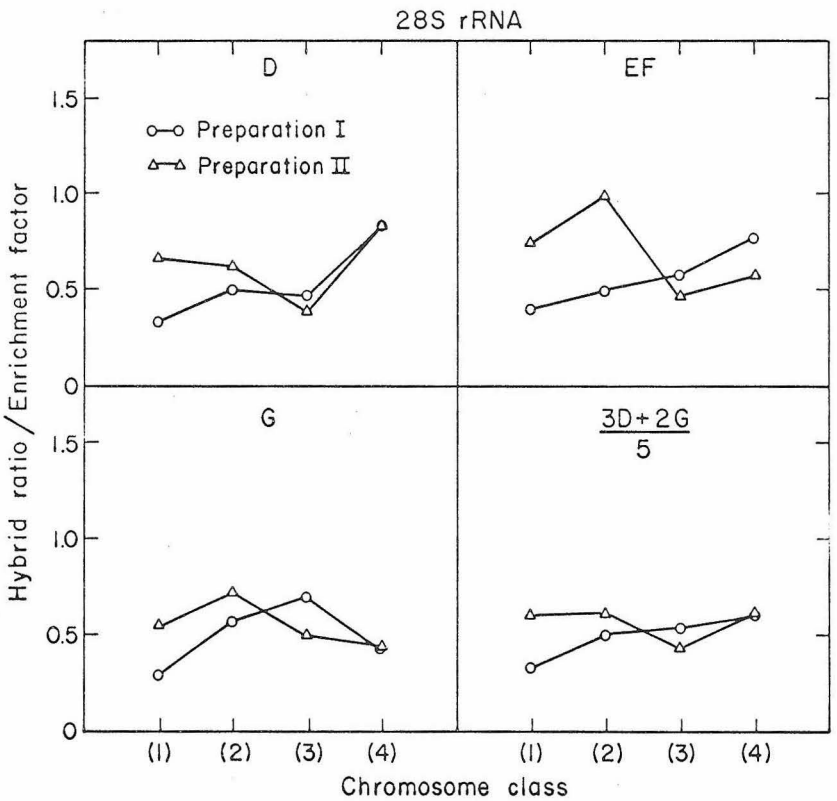

28S rRNA
D
EF
G
3D+2G
5
Preparation I
Preparation II
Hybrid ratio / Enrichment factor
1.5
1.0
0.5
0
(1)
(2)
(3)
(4)
Chromosome class

Figure 3a.

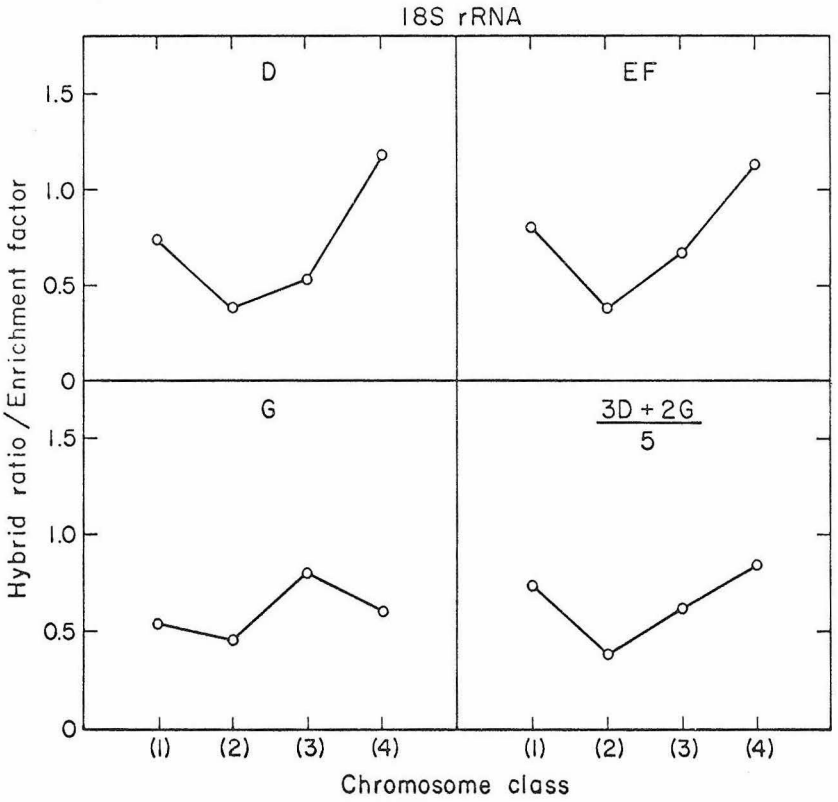

18S rRNA
D
EF
G
3D + 2G
5
1.5
1.0
0.5
0
Hybrid ratio / Enrichment factor
(1)
(2)
(3)
(4)
Chromosome class

Figure 3b.

$\frac{3D + 2G}{5}$ and other groups in the various chromosome classes. Indeed, the fact that the quantity

$$\frac{\text{hybrid ratio}}{\text{enrichment factor}}$$

should be independent of chromosome preparation for the group or combination of groups containing RNA sites and may vary with chromosome preparation for irrelevant groups is another criterion which can be used to help localize RNA sites.

(ii) The reduced capacity of chromosomal DNA for hybridization with rRNA.

There are several possible explanations for the apparent reduction in hybridization capacity of DNA from isolated and fractionated chromosomes. One is that the proportion of rRNA sites in DNA extracted from metaphase chromosomes might be less than that of DNA extracted from interphase cells. To test this possibility we have extracted DNA from whole vinblastine sulfate-treated HeLa cells (75% metaphase) and tested the hybridization capacity of this DNA with rRNA. Such DNA hybridizes as well as DNA from untreated HeLa cells. We have also prepared DNA from interphase nuclei (isolated by the normal chromosome isolation procedure and then centrifuged through a glycerol-sucrose gradient). This interphase nuclear DNA also hybridizes as well as DNA from whole HeLa cells.

Another possibility is that the rRNA sites might be contained, at least partially, in structures which could be selectively removed from the chromosomes during chromosome isolation and fractionation. In this case the selectively lost rRNA sites should be recoverable in one of those fractions of cell debris separated from the chromosomes during

isolation and fractionation. To test this possibility we have prepared DNA from all such fractions. These fractions are:

1. Debris contained in the supernatant of the 900 g centrifugation which follows homogenization.
2. Debris which does not pellet through 2.2M sucrose.
3. Debris (mostly nuclei and chromosome aggregates) which sediments more rapidly than chromosome class (1) during fractionation.
4. Debris which sediments more slowly than chromosome class (4) during fractionation.

Most, if not all, of the DNA isolated from these fractions can be accounted for by chromosomal and nuclear contamination.

DNA samples from all the above fractions hybridize with rRNA to the extent one would predict on the basis of their contamination by nuclei and D and G chromosomes. Certainly none of them hybridizes nearly well enough to account for the "lost" rRNA sites.

A third possibility is that during the course of isolation and fractionation the DNA of the metaphase chromosomes may be altered in such a way that its efficiency of hybridization is reduced.

This is also unlikely. In fact, earlier experiments (Huberman & Attardi, 1966) which showed that DNA from isolated chromosomes has the same base composition (within 1%) as DNA from whole HeLa cells suggest that alterations in base composition probably cannot explain the reduced hybridization capacity. Since the hyperchromic effects produced by denaturation are identical for whole cell DNA and chromosomal DNA,

differences in denaturability are probably not a satisfactory explanation. In addition, contamination of the DNA preparations by protein probably cannot account for the reduced hybridization capacity, for extensive further treatment of the chromosomal and whole cell DNA preparations with phenol and with pronase does not alter their hybridization capacities. Finally, differences in size distribution for single strands of DNA from isolated chromosomes and whole cells probably cannot account for the difference in hybridization capacity, either. We have observed that DNA samples prepared either from isolated, fractionated chromosomes or from whole HeLa cells, and then heat-denatured, sediment in alkaline (pH 12.6) CsCl density gradients (Vinograd, Bruner, Kent & Weigle, 1963) as symmetrical but rather broad bands and that the band centers for both kinds of DNA have identical S values.

(c) Hybridization with cytoplasmic mRNA

The RNA used in the experiments to be described was prepared from the cytoplasmic polysome fraction of HeLa cells pulse-labeled for 45 min with ^{3}H-uridine. The major labeled component in the polysomes after such pulses is mRNA (Penman, Scherrer, Becker & Darnell, 1963). However, an appreciable amount of radioactivity is also associated with 18S rRNA (Penman, 1966). Therefore, in pooling the components in the sucrose gradient pattern of the RNA corresponding to S values between 7 and 40, the peak fractions for 18S RNA were excluded. Due to the presence of large amounts of unlabeled RNA, the specific activity of the preparation was only 400 cts/min/μg. On the basis of the known rRNA site content

of HeLa DNA, rRNA with a specific activity of about 25,000 cts/min/μg would have been required to obtain hybrid values comparable to those actually obtained (see below); thus it is evident that the small amount of residual labeled rRNA did not contribute to the results.

All the experiments described here were conducted at a ratio of total RNA:DNA of 0.4. This ratio presumably corresponds to a ratio of mRNA:DNA of 0.01-0.02 (Latham & Darnell, 1965) which is far below that required for saturation of all mRNA sites. We assumed that hybrid values obtained under such conditions would still be proportional to the fraction of DNA complementary to cytoplasmic mRNA.

The method of hybridization used was similar to that for rRNA (see Materials and Methods). In all cases, hybrid values were determined as the amounts of labeled RNA retained on membrane filters. Our results are expressed as hybrid ratios. The hybrid value obtained with whole HeLa cell DNA in various experiments was 5-7 cts/min/μg DNA. An estimate of the maximum non-specific background was obtained by using E. coli DNA, which gave a value of 0.5 cts/min/μg DNA.

Hybrid ratios for hybridization between cytoplasmic mRNA and DNA from chromosome preparations I and II are shown in Table 5.

It is evident that DNA samples from the different chromosome classes give approximately the same hybrid ratios. Thus mRNA sites are evenly distributed among chromosomes of all sizes. It is also apparent that, as in the case with rRNA, the capacity for hybridization with cytoplasmic mRNA is reduced for the DNA from the chromosome preparations (the hybrid ratios are all less than 1).

TABLE 5

Results of Hybridization between Cytoplasmic mRNA and Chromosomal DNA

Chromosome preparation	Class number	Hybrid ratio
I	(1)	0.4
	(2)	0.3
	(3)	0.3
	(4)	0.4
II	(1)	0.4
	(2)	0.4
	(3)	0.5
	(4)	0.5

Class numbers and hybrid ratios are defined in the text. Hybridization experiments were performed as described in Materials and Methods.

A preliminary investigation of the possible causes of this reduction of hybridization capacity has been conducted. As in the case with rRNA, DNA extracted from whole vinblastine sulfate-treated cells (75% metaphase) hybridizes with mRNA as well as does whole cell DNA. Likewise, extensive phenol and pronase treatments do not alter the relative capacities of chromosomal and whole cell DNA for hybridization with mRNA.

Unlike the case with rRNA, however, interphase nuclear DNA has an mRNA hybrid ratio of 0.4. Furthermore we have observed that if, during the course of chromosome isolation, centrifugation through 2.2 M sucrose is omitted, DNA from the resulting chromosome preparation has an mRNA hybrid ratio of 1.0 (in contrast,hybridization with rRNA is the same as for DNA from purified chromosome preparations). In addition, DNA isolated from the debris which normally does not sediment through 2.2 M sucrose has an mRNA hybrid ratio of 1.5. Although this hybrid ratio is at least threefold greater than that given by DNA from purified chromosomes, the amount of DNA that does not pass through 2.2 M sucrose is such that a hybrid ratio of 5 or more would be required to account for the difference between DNA from purified chromosome preparations and DNA from preparations not passed through 2.2 M sucrose. This discrepancy will be examined in the Discussion section.

4. DISCUSSION

(a) Chromosomal distribution of 28S and 18S rRNA sites

In the last few years evidence has been accumulated that rRNA sites are located in the nucleolar organizer regions of chromosomes. Ritossa

and Spiegelman((1965) have shown conclusively that all the rRNA sites, or at least the controlling elements of their production, in Drosophila melanogaster are confined to a small chromosome region identical or close to the nucleolar organizer. Similarly, Wallace and Birnstiel (1966) have demonstrated a direct proportionality between the number of rRNA sites and the number of nucleolar organizers in Xenopus laevis. In the case of HeLa cells the location of rRNA sites has been less clear. The experiments of McConkey and Hopkins (1964) demonstrated an obvious enrichment of 28S rRNA sites in the nucleolus-associated DNA of HeLa cells, but this DNA could only account for about 1/7 of the total 28S rRNA sites.

Our results show that at least 50% of the rRNA sites of HeLa cells are confined to chromosomes of groups D, E, F, and G, and they suggest a possibly exclusive localization of these rRNA sites to the D and G chromosomes. Since the available cytological evidence (Ohno, Trujillo, Kaplan & Kinosita, 1961; Ferguson-Smith & Handmaker, 1963; Yerganian, 1963; Ferguson-Smith, 1964) indicates that most, if not all, nucleolar organizer activity is confined to chromosomes of groups D and G, our results are certainly consistent with the concept that all rRNA sites are located in nucleolar organizer regions.

It should be pointed out that our results also imply that the rRNA sites are a part of the smaller chromosomes and are not extra-chromosomal. Both the Ritossa & Spiegelman (1965) experiment and the Wallace & Birnstiel (1966) experiment could conceivably have been

interpreted as showing that the nucleolar organizer regions controlled the production of extrachromosomal copies of a few chromosomal sites. Indeed, Miller (1964) and Kezer (results cited by Peacock, 1965) have shown that the nucleolar organizer region of salamander lampbrush chromosomes can be duplicated many times in an extrachromosomal form. Lampbrush chromosomes are found only in oocytes, however. To test the possibility that normal tissues might also contain extrachromosomal rRNA sites, Ritossa, Atwood, Lindsley & Spiegelman (1966) have examined the rRNA site content of DNA samples from various chicken tissues having different levels of rRNA synthesis. Their finding, that all the tissues investigated have the same rRNA site content per unit DNA, tends to support a true chromosomal location for rRNA sites. However, the possible presence of extrachromosomal rRNA sites, even in tissues not actively synthesizing rRNA such as sperm cells and erythrocytes, cannot be ruled out. Our findings are less ambiguous in this respect. Our hybridization results with DNA from isolated chromosomes show directly that at least 50% of the rRNA sites of the cell are chromosomal. Our failure to find rRNA sites in other cell fractions than chromosomes or nuclei suggests that HeLa cells contain no cytoplasmic extrachromosomal rRNA sites. Furthermore, the fact that DNA from whole vinblastine sulfate-treated cells (75% metaphase) hybridizes with rRNA as well as does DNA from untreated cells suggests that interphase nuclei contain no greater proportion of rRNA sites than do metaphase chromosomes and thus that there are no extrachromosomal rRNA sites in HeLa nuclei either.

(b) Chromosomal distribution of cytoplasmic mRNA sites

Our results suggest that chromosomal sites for cytoplasmic mRNA are distributed more or less uniformly on chromosomes of all sizes. This conclusion is an agreement with current concepts on the heterogeneity of cytoplasmic mRNA.

(c) The reduced hybridization capacity of chromosomal DNA

There are four possible explanations for the observed reduction in hybridization capacity of chromosomal DNA. These are:

1. Selective loss of m- or rRNA sites during isolation and fractionation of chromosomes.

2. Selective loss of m- or rRNA sites during extraction of DNA from chromosomes.

3. Early replication of m- or rRNA sites resulting in a greater proportion of such sites in DNA from non-synchronized exponentially growing cells than in DNA from metaphase cells.

4. Physical or chemical alteration, during isolation and fractionation, of the chromosomal DNA resulting in generally reduced hybridization efficiency.

In the case of rRNA, our controls tend to rule out the first, third, and fourth explanations. The second explanation, which seemed a priori attractive because of the known structural differentiation of nucleolar organizer regions (secondary constrictions), is not completely excluded, however. The fact that DNA from whole metaphase cells hybridizes with

rRNA as well as does DNA from whole normal cells shows only that rRNA sites are not selectively lost during DNA extraction from native metaphase chromosomes in whole cells. The possibility remains that rRNA sites could be selectively lost during extraction of DNA from isolated metaphase chromosomes.

In the case of mRNA, our controls suggest that the first explanation, selective loss of mRNA sites during purification of the chromosomes, is the most plausible. Recent experiments by Attardi and Attardi (1967) help to explain how such a loss could arise. They have discovered in HeLa cells a fraction of rapidly-labeled cytoplasmic RNA which is bound to membranes and which is apparently synthesized on a cytoplasmic, rather than a chromosomal, DNA template. This RNA is capable of hybridizing with cytoplasmic DNA (presumably mitochondrial) with an extremely high efficiency.

The results of Attardi and Attardi (1967) suggest that preparations of cytoplasmic mRNA made as described in Materials and Methods probably contain a small amount of such cytoplasmic membrane-bound RNA.

We have shown that DNA prepared from chromosomes which have not been purified by centrifugation through 2.2 M sucrose has an mRNA hybrid ratio of 1.0 while DNA from purified chromosomes has an mRNA hybrid ratio of 0.3 - 0.5. It seems likely that both the DNA from unpurified chromosomes and DNA from whole cells contain a small amount of DNA of cytoplasmic origin. It is possible, then, that highly efficient hybridization between this cytoplasmic DNA and the small amount of membrane-bound RNA contaminating our mRNA preparations contributed

one-half to two-thirds of the total amount of hybrid detected when using either whole cell DNA or DNA from impure chromosomes. The low mRNA hybrid ratios given by DNA from pure chromosomes or pure isolated nuclei can be explained by the absence of contaminating cytoplasmic DNA. The fact that DNA from debris which did not pass through 2.2 M sucrose gave an mRNA hybrid ratio of only 1.5 while a hybrid ratio of 5 or greater would have been required to account for the difference between pure and impure chromosome preparations can be explained by exhaustion of the small amount of membrane-bound RNA in the mRNA preparations.

(d) The value of chromosome fractionation

Results presented in this paper show that the present technique for chromosome fractionation is capable of resolving, in a usable if not pure form, the major morphological groups of the human karyotype. Undoubtedly future technical advances will improve this resolution. One may also anticipate that future improvements will allow elimination, or at least understanding, of the variations in hybridization capacity produced by isolation or fractionation of chromosomes.

We feel that the potential usefulness of the fractionation technique even in its present form warrants a brief survey of obvious applications. Certainly a test of the chromosomal distribution of sites for the other classes of readily available RNA (5S rRNA and 4S transfer RNA) should be possible, and such a study is now being carried out in our laboratory. Furthermore we can see no insurmountable obstacles to similar tests

using specific types of mRNA, provided sufficient quantities of specific mRNA can be isolated and that the sensitivity of hybridization techniques can be improved. In addition, recent experiments by Britten and co-workers (Britten & Kohne, 1966: Waring & Britten, 1966) have demonstrated the large-scale existence of redundant sequences in the DNA of higher organisms. The chromosomal distribution of such redundant DNA could probably be determined by chromosome fractionation techniques.

One might hope, eventually, to be able to use chromosome fractionation as a step in the purification of gene-specific chromosomal proteins or gene-specific chromosomal RNA. For most such applications, though, considerably higher resolution will be required. Meanwhile, a search for large-scale differences in protein or RNA content between chromosomes of different groups could lead to interesting and rewarding results.

We are indebted to Roger Radloff for assistance with CsCl density gradient centrifugation and to Baldomero Olivera for assistance with electrophoresis. We gratefully acknowledge the help of Thomas Hazlet in part of this work and the valuable technical assistance of Benneta Keeley and La Verne Wenzel. This investigation was supported by the U.S. Public Health Service, grant numbers GM 11726 and 5-F1-GM-21,622.

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PART II

AUTORADIOGRAPHIC STUDIES OF THE STRUCTURE AND REPLICATION OF MAMMALIAN CHROMOSOMAL DNA

INTRODUCTION

The studies described in Part II are the result of a collaboration between Arthur D. Riggs and myself which began in the spring of 1965. We both agreed that a great deal more could be learned about the size, and perhaps the replication, of chromosomal DNA molecules by application to higher organisms of the technique developed by Cairns (1) for autoradiographic visualization of bacterial DNA molecules. For our first experiments we tried autoradiography, by Riggs' (2) modification of the Cairns technique, of DNA from Chinese hamster cells grown in tissue culture. These experiments were successful, and their results are reported in Chapter I. Our main finding was that we could detect DNA fibers up to 1,800 μ long.

As pointed out in the discussion of Chapter I, our finding of such long DNA fibers did not definitively settle any questions about chromosome structure. However, it did allow us to ask further questions about the replication of chromosomal DNA. The questions we asked, and the answers we obtained, are reported in Chapter II.

CHAPTER I

Autoradiography of Chromosomal DNA Fibers from Chinese Hamster Cells

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*AUTORADIOGRAPHY OF CHROMOSOMAL DNA FIBERS FROM CHINESE HAMSTER CELLS**

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Communicated by Norman Davidson, January 24, 1966

Ignorance of the true length of the DNA molecules in the chromosomes of higher organisms has always been a major obstacle to understanding chromosome structure. Consequently, attempts have been made, usually with the aid of electron microscopy, to estimate the size of DNA in higher organisms. Solari[1] has reported the longest such DNA measured before now—a DNA fiber from a sea urchin sperm at least 93 μ long.

The autoradiographic technique developed by Cairns[2] for visualizing DNA has allowed measurement of fibers much longer than 93 μ. It has been used successfully by Cairns[2] with bacterial DNA, and by Riggs and Mitchell[3] with DNA from PPLO. This paper presents the results we obtained by applying the Cairns technique to Chinese hamster cells grown in tissue culture.

Methods.—Incorporation of H^3-thymidine. Cells of Chinese hamster fibroblast strain B14FAF28 (a gift from Dr. T. C. Hsu) were grown as monolayer cultures on plastic Petri dishes in Eagle's medium supplemented with 10% calf serum. At a cell density of 10^5 cells/ml of medium, 5-fluorodeoxyuridine (FUDR, courtesy of Hoffmann-LaRoche Laboratories, Inc.), an inhibitor of thymidine biosynthesis, was added to make 0.05 μg/ml. Uridine was added to 2.5 μg/ml at the same time. About 10 hr later H^3-thymidine (14 c/mmole, New England Nuclear Corp.) was added to 4 μg/ml. Incubation was continued for 35–40 hr. Then the cells were harvested by a 10-min treatment at 37°C with 0.05% trypsin in TD (0.137 *M* NaCl, 0.005 *M* KCl, 0.007 *M* NaH_2PO_4, 0.025 *M* tris, pH 7.4, containing 100 mg/liter of streptomycin sulfate and 5×10^5 units/liter of penicillin G) and diluted to about 400 cells/ml in TD.

Lysis and spreading procedure: The method usually used was a modification by Riggs and Mitchell[3] of the procedure developed by Cairns.[2] The cells suspended in TD were diluted tenfold into "lysis medium" (1.0 *M* sucrose, 0.05 *M* NaCl, 0.01 *M* EDTA, pH 8.0). Usually calf thymus DNA was added at this point to 5 μg/ml. One ml of cell suspension (about 40 cells) was then introduced through a polyethylene tube into a dialysis chamber. Construction of the dialysis chambers is outlined in Figure 1. The cells were lysed by dialysis for 3 hr at 34°C against 250 ml of 1% sodium dodecyl sulfate (SDS) in "lysis medium." Further dialysis (6 changes of 2 hr each) against "dialysis medium" (0.05 *M* NaCl, 0.005 *M* EDTA, pH 8.0) served to remove SDS and unincorporated thymidine. Finally, the dialysis chambers were removed from the "dialysis medium" and emptied, either by draining through a small hole pierced in one of the VM filters, or by siphoning through the glass inlet tube. In the process of emptying, some DNA was trapped on the VM filters and was spread out as the liquid meniscus moved past.

Single cell method: In some cases, cells were suspended in "lysis medium" at an average concentration of 0.5 cell/μl. Drops of 2 μl were placed (one drop per filter) on VM filters which had been coated with a thin film of silicone grease around the outer edge and soaked in "lysis medium." The drops were examined microscopically. Those drops containing single cells were diluted to 0.1 ml with "lysis medium." The drops, still on the filters, were dialyzed first against 1% SDS in "lysis medium" (3 hr) and then against "dialysis medium" (6 changes of 2 hr each) by floating the filters on the surface of the appropriate solutions. The liquid remaining on top of each filter was drained off through the filter by transferring the filter to a dry surface and then placing the point of a wedge-shaped piece of bibulous paper underneath its center. In this way all DNA was trapped on the filter.

Pronase digestion: In some cases, after the fifth change of dialysis medium, dialysis was continued for 14.5 hr against SSC-tris (0.15 *M* NaCl, 0.015 *M* trisodium citrate, 0.01 *M* tris, pH 8.0) containing pronase (Calbiochem, B grade) at a concentration of 50 μg/ml. Under these condi-

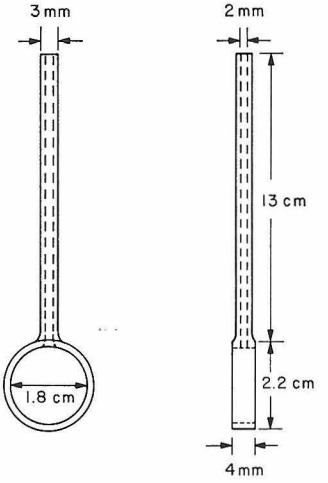

FIG. 1.—Dialysis chamber. A 3-mm OD Pyrex tube was fused to a 2.2-cm OD Pyrex tube. The large tube was then cut to form a thin cylinder with an arm for filling and handling. Using plastic dissolved in amyl acetate as a glue, a VM Millipore filter (50 mμ average pore size, Millipore Filter Corp., Bedford, Mass.) 25 mm in diameter was glued to each end of the glass cylinder.

tions, there was no detectable nuclease activity in this lot of pronase.[31] Some of the pronase was probably adsorbed by the Millipore filters. Hence, after pronase treatment, the contents of the dialysis chambers were always collected by siphoning and then assayed for pronase activity. The assay medium contained SSC-tris and 5 mg/ml of casein. Incubation was carried out at 37°C for 0.5–14.5 hr, and then trichloroacetic acid was added to a final concentration of 5%. Precipitated material was removed by centrifugation, and the optical density of the supernatant was determined at 280 mμ. An OD_{280} of 1.61 corresponded to the conversion of 5 mg/ml of casein to acid-soluble form.

Autoradiography: The filters were allowed to dry thoroughly, and were then cut from the dialysis chambers, glued to glass microslides, and covered with Kodak AR-10 Autoradiographic Stripping Film (Eastman Kodak Co.). The slides were placed in lightproof boxes along with some $CaSO_4$ drying agent and exposed for 1–4 months in a CO_2 atmosphere at −15°C. At the end of this period, the film was developed in Kodak D-19b at 20°C for 20 min. After development the stripping film was peeled from the slide to which the filter had been glued and mounted on a new, clean slide with Permount and a cover glass.

Observation: The slides were usually scanned with a microscope at 100×, using dark-field optics to increase contrast. Most photographs were taken at 40× with dark field. The lengths of individual DNA autoradiograms were determined from photographic enlargements using a map measurer. For grain counts, photographs of the area of interest were taken at 400× with phase optics, enlargements were made, and divisions corresponding to 4 μ were marked off along an imaginary line interpolated through the grains of the autoradiogram being examined. All grains in each interval were counted if they fell within a distance corresponding to 1.5 μ of the imaginary line.

To obtain length distributions, the image produced by a microscope with dark-field optics was projected onto a ground-glass screen. All DNA autoradiograms in each field of view were then traced onto tracing paper. Hundreds of autoradiograms, corresponding to areas of one third of a Millipore VM filter or more, could readily be traced in this way. Lengths were determined from the tracings with a map measurer.

Results.—Observed distribution of lengths: In describing our results we shall use the word "autoradiogram" to refer to any apparently continuous line of grains presumably caused by decay of tritium incorporated into DNA. We shall also use the word "fiber" to refer to any single thread consisting of DNA and other substances associated with DNA. The DNA in a single fiber need not be a single molecule.

The basic experiment reported here has been repeated four times, and the results of all experiments are in agreement.

Figure 2 shows an autoradiographic field from one of the more concentrated areas on a filter. The longest DNA autoradiogram visible (arrows) is 1.1 mm long, and there are many shorter autoradiograms. The distribution of lengths observed on a filter chosen for the clarity and good spreading of its DNA autoradiograms is given in Figure 3. One can see that the most frequent autoradiograms are shorter than 0.1 mm. However, 6 per cent of the autoradiograms are longer than 0.8 mm. Figure 4 shows the distribution obtained when the same data are weighted accord-

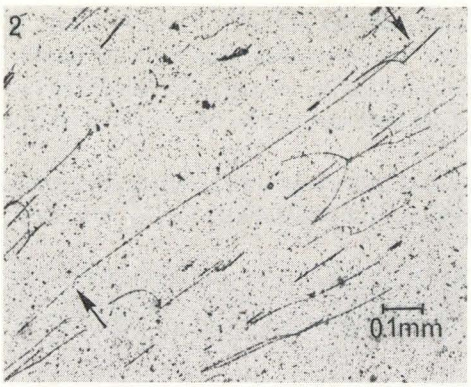

FIG. 2.—Typical DNA autoradiograms. Arrows indicate long autoradiogram. Exposure time was 3 months.

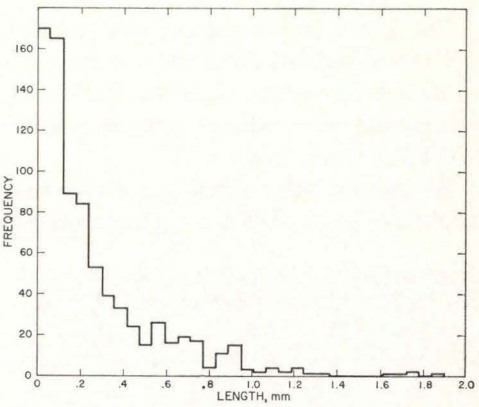

FIG. 3.—Frequency of DNA autoradiograms as a function of length.

ing to length. It is apparent that 50 per cent of the total length of autoradiograms is accounted for by autoradiograms equal to or longer than 0.5 mm.

The longest autoradiograms we observe are of special interest. Figure 5 shows a very long autoradiogram which we have used for grain count studies and two more long autoradiograms are shown in Figure 6.

The significance of these autoradiograms depends on the answers to four questions: (*a*) Were the autoradiograms produced by Chinese hamster cell DNA? (*b*) If so, was the DNA of chromosomal or extrachromosomal origin? (*c*) Are the autoradiograms the same length as the DNA producing them or are they distorted? (*d*) Were they produced by single DNA fibers or by unnatural aggregates of DNA?

That the autoradiograms were produced by DNA is suggested by the fact that no autoradiograms were found if the cells were not lysed. Consequently, the autoradiograms must have been produced by some cell component; they could not be due to scratches on the stripping film (scratches were sometimes observed, but they could be distinguished from DNA autoradiograms). The only known cell component of such size into which thymidine is incorporated is DNA.

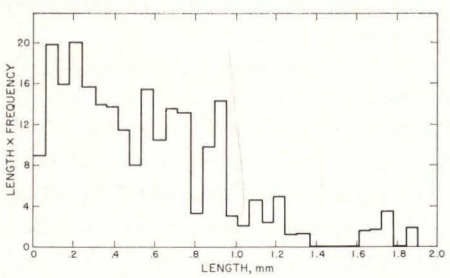

FIG. 4.—Frequency times length of DNA autoradiograms as a function of length. Data are the same as in Fig. 3.

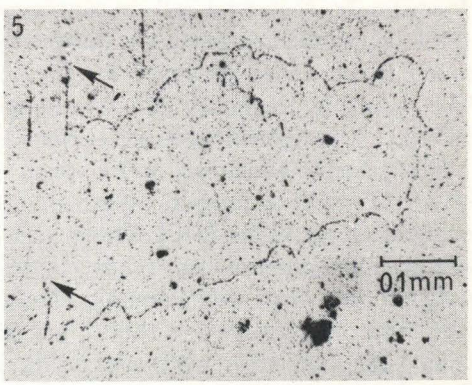

FIG. 5.—A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was 3 months.

The DNA producing the autoradiograms was almost certainly Chinese hamster cell DNA and not DNA from a contaminating microorganism, for we were unable to detect any contamination. Microscopic examination of the cells used for the experiments showed no microorganisms, and tests of the stock culture for bacteria and PPLO were negative.[4]

We cannot rule out the possibility that a small proportion of the autoradiograms might be due to DNA of extrachromosomal (e.g., mitochondrial) origin, but most

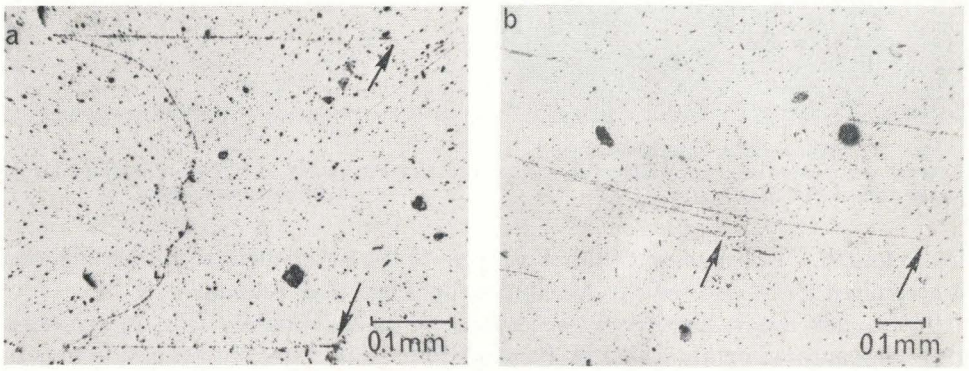

FIG. 6.—(*a*) A DNA autoradiogram 1.4 mm long (between arrows). Exposure time was 3 months. (*b*) A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was $3^1/_2$ months.

of them were certainly produced by chromosomal DNA since at least 7 per cent of the DNA in a single Chinese hamster cell is required to account for all the autoradiograms produced by such a cell. This estimate, which was made by our single cell technique (see *Methods*), is certainly too low because a great deal of the DNA from single cells was tangled and produced unmeasurable autoradiograms. Nevertheless, it is highly unlikely that extrachromosomal DNA could, by itself, account for even 7 per cent of the DNA in a single cell. Furthermore, in our first experiment it was necessary to calculate the proper number of cells to put in the dialysis chambers. In making this calculation we assumed that *all* the DNA in the cells would (*a*) be released from the cells and (*b*) produce autoradiograms if trapped on a filter. Accordingly, we used a cell concentration (20 cells/ml) just high enough to give a DNA concentration inside the dialysis chambers (2×10^{-4} μg/ml) that had been found to be about optimum for the Cairns procedure when applied to PPLO.[3] The resulting frequency of autoradiograms was similar to that obtained with PPLO. This agreement suggests that our assumptions were correct.

The possible measuring errors which could lead to large overestimates of length are: (1) errors in magnification and in measurement of the autoradiograms; (2) overstretching of the DNA fibers; and (3) stretching of the stripping film. The first possible source of error can be eliminated because both the magnification and the measurement steps were carefully checked. The combined error of the magnification steps was always less than 3 per cent. The map measurer we used had less than 1 per cent error when measuring straight lines, and even for highly twisted lines the reproducibility was better than 5 per cent.

The second and third possibilities combined are unlikely to lead to errors greater than about 25 per cent, because the same procedure gives values for the *E. coli* chro-

mosome with a variation from the mean of less than 12 per cent[2] and these values are in agreement with independent determinations.[2] In addition, the area of the stripping film that covered the Millipore filter could usually be distinguished after processing of the film was completed, and this area remained the same size and shape as the filter it had covered. Thus, no net stretching of the film occurred. Also, the linear grain density produced by abnormally stretched fibers should be much less than the average linear grain density. This was not the case for the great majority of long autoradiograms seen.

Several lines of argument support the conclusion that most of the DNA which produced our autoradiograms consisted of single fibers rather than aggregates. We counted grains in 4-μ intervals over entire long autoradiograms (see *Methods*), and we found that the number of grains per interval followed a Poisson distribution. Table 1 shows the grain count data from the autoradiogram in Figure 5. Overlaps or discontinuities in the DNA fibers responsible for the autoradiograms would have produced deviations from the Poisson distribution, provided that such abnormalities extended for a few microns or more. The low concentration of labeled DNA present in the dialysis chambers (usually 4.0×10^{-4} μg/ml) also is an argument against the possibility of aggregation of labeled DNA fibers. Furthermore, neither varying the concentration of labeled DNA from 2.0 to 20 $\times 10^{-4}$ μg/ml nor omitting the calf thymus DNA from the dialysis chambers had any significant effect on the autoradiogram lengths.

TABLE 1

GRAIN COUNT DATA

No. of grains per 4-μ interval	Observed frequency	Expected frequency
0	13	12.1
1	39	42.6
2	69	75.1
3	88	88.5
4	86	78.0
5	62	55.3
6	35	32.4
7	13	16.4
8	6	7.2
≥ 9	1	4.5

Data are taken from the autoradiogram shown in Fig. 6*b*. The expected frequency is calculated, using the Poisson formula and the mean number of grains per interval (3.53). $\chi^2 = 6.3$, which is well under the rejection limit of 13.4 at the 10% level for 8 degrees of freedom.

In summary, the autoradiograms are unexaggerated representations of single fibers of Chinese hamster DNA, mostly or entirely of chromosomal origin. Consequently, they are significant as representations of the longest apparently continuous DNA fibers yet reported for higher organisms. Indeed, our longest autoradiograms are more than 15 times longer than the DNA fiber reported by Solari,[1] and they are even somewhat longer than the 1.1–1.4 mm reported by Cairns[2] for the *E. coli* chromosome. Since 1 mm of DNA has a molecular weight of about 2×10^9 daltons, our longest autoradiograms represent $3.2–3.6 \times 10^9$ daltons of DNA.

Effect of pronase on autoradiogram lengths: We have performed one successful experiment in which the contents of some dialysis chambers were dialyzed against pronase in SSC-tris for 14.5 hr (see *Methods*) after the cells had been lysed by SDS. Controls were dialyzed against SSC-tris for the same length of time. The pronase activity inside the dialysis chambers at the end of dialysis was sufficient to solubilize 0.14 mg of casein per hour under the conditions of the assay. The longest autoradiograms from the pronase-treated filters were just as long as those from the controls. We conclude tentatively that the DNA fibers producing the autoradiograms do not contain linkers readily susceptible to pronase under the con-

ditions we employed. To date, experiments with higher concentrations of pronase have failed because pronase attacks the stripping film.

Discussion.—We do not yet have enough information to establish definitely the relationship between the autoradiograms we observe and the DNA molecules in chromosomes. It is possible that the chromosomal DNA molecules are shorter than our autoradiograms. If so, the molecules must be joined tandemly (by linkers of another substance) to form fibers at least as long as our autoradiograms, and the linkers must be resistant to both SDS and pronase under the conditions employed.

On the other hand, the DNA molecules could be longer than the autoradiograms for many reasons. The cells may be incompletely lysed during preparation or the DNA fibers may not be completely untangled. Even if the fibers are properly untangled, they may be incompletely stretched out or they may have contracted when drying. Portions of the fibers may be held away from the stripping film in the pores of the Millipore filter. The fibers may be partially degraded by nuclease action, by mechanical shear, or by tritium decay, and, finally, the fibers may not be completely labeled. Further experiments are required to test these possibilities.

It is not unlikely, however, that some of the autoradiograms we observe may be close to the true length of chromosomal DNA molecules. There is now considerable evidence that individual chromosomes of higher organisms contain many independent DNA replication points, as discussed below. The existence of multiple replication points can be explained most simply in terms of independently replicating DNA molecules, and several criteria suggest that these hypothetical DNA molecules should be about the size of our longer autoradiograms.

Evidence for multiple replication points comes from numerous experiments, with both animal[5–21] and plant[22, 23] cells, which show that tritiated thymidine can be incorporated into many separate sites in single chromosomes after pulses which are short compared to the time required for complete DNA replication. Furthermore, the giant chromosomes of *Drosophila* are sufficiently extended so that the separate incorporation points can sometimes be counted. Plaut and Nash[24] find up to 50 incorporation points per *Drosophila* chromosome, but they consider the true number to be higher.

The tremendous total length of DNA in the chromosomes of higher organisms also suggests that chromosomes must contain many replication points. Even at the fast bacterial rate of DNA synthesis (up to 100 μ per min[2]), 15 hr would be required to replicate all the DNA (about 9 cm[25]) of an average Chinese hamster chromosome if there were one replication point per chromosome. Total DNA synthesis takes only about 6 hr in these cells.[6, 20] Furthermore, the heterochromatic X chromosome of female Chinese hamster cells, one of the larger chromosomes, is known to replicate in about 1.5 hr.[20] Other animals, too, synthesize DNA more rapidly than would be expected on the basis of a single replication point per chromosome. Early cleavages in the embryos of many invertebrates occur at intervals of less than 30 min,[26] and for the first 10–12 divisions after fertilization in *Drosophila* the entire mitotic cycle takes less than 10 min.[27]

If the assumption is made that each DNA replication point in a chromosome corresponds to one DNA molecule, then it should be possible to estimate the average length of chromosomal DNA molecules. An estimate of the number of replication points per chromosome has been made only for *Drosophila* (Plaut and Nash[24]). If

this estimate (50) is divided into the average *Drosophila* chromosomal DNA content of 1.5–7.5 cm,[28] a length of 0.3–1.5 mm for an average chromosomal DNA molecule is obtained.

If, in addition, the rate of DNA synthesis in Chinese hamster cells is assumed to be the same as that in *E. coli*, then the maximum possible length for DNA molecules in the heterochromatic X chromosome can be estimated directly as 9 mm. Since all the molecules of the heterochromatic X may not replicate at once, 9 mm is probably an overestimate.

These estimates of chromosomal DNA length in Chinese hamster cells and in *Drosophila* are of the same order of magnitude as our longer autoradiogram lengths. This agreement suggests that it is possible that some of our autoradiograms represent whole chromosomal DNA molecules. If so, then the bonds holding the molecules together in the chromosome may be sensitive to SDS.

We also do not have enough information to establish the arrangement of DNA molecules in chromosomes. However, we can make some preliminary conclusions. Our knowledge of genetics suggests that, on a large scale, DNA molecules are arranged in linear sequence but that circularity of individual molecules is possible.[29] We find no evidence for circular molecules in our autoradiograms. Although we find no evidence against the possibilities that our autoradiograms either represent fragments of originally larger circular molecules or represent molecules once held in circular configuration by SDS-sensitive bonds, circles shorter than about 1 mm in circumference and without SDS-sensitive bonds probably could not have produced our autoradiograms. In particular, our results cannot be easily explained in terms of small circles of the type recently reported by Hotta and Bassel[30] for DNA from boar sperm.

Summary.—Linear DNA autoradiograms are found when the Cairns technique is applied to Chinese hamster cells. At least 6 per cent of these autoradiograms are more than 0.8 mm long—roughly the size of the *E. coli* chromosome and considerably longer than previously reported DNA fibers from higher organisms. Some rare autoradiograms are as long as 1.6–1.8 mm. The implications of these results in terms of models of chromosome structure are discussed.

We gratefully acknowledge the advice and assistance of Dr. Giuseppe Attardi and of Dr. Herschel K. Mitchell, in whose laboratories this work was done.

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FURTHER DISCUSSION

Since the time this report appeared, a great deal more has been learned. Cairns (3) and Sasaki & Norman (4) have published the results of similar autoradiographic experiments with mammalian DNA, and we have continued our own experiments. I shall use this new knowledge to discuss the probable size of the DNA fibers in mammalian chromosomes. First, however, it will be necessary to consider the results of our more recent autoradiographic experiments (Chapter II, publication).

CHAPTER II

On the Mechanism of DNA Replication in Mammalian Chromosomes

The first part of this chapter has been submitted for publication in the Journal of Molecular Biology. It is included here in the form in which it was submitted.

On the Mechanism of DNA Replication in Mammalian Chromosomes

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SUMMARY

We have combined the techniques of pulse labeling and DNA autoradiography to investigate the mechanism of DNA replication in the chromosomes of Chinese hamster and HeLa cells. Our results prove that the long fibers of which chromosomal DNA is composed are made up of many tandemly-joined replication units in which DNA is probably replicated at fork-like growing points. In Chinese hamster cells most of these units are probably less than 60 μ long, and the rate of DNA replication per growing point is 2.5 μ/min or less.

In addition, we have taken advantage of the apparent slowness of equilibration with external thymidine of the internal thymidine triphosphate pool in Chinese hamster cells to determine the direction of DNA synthesis at the conclusion of the pulse in pulse-chase experiments. Our finding, that replication seems to proceed in opposite directions at adjacent growing points, suggests that replication may start in the interior of each replication unit and proceed outwards, at two growing points, to the ends of the replication unit.

1. Introduction

These studies were undertaken in the hope of increasing our understanding of the mechanism by which the large amount of DNA in the chromosomes of higher organisms is replicated. Previous autoradiographic experiments (Cairns, 1966; Huberman & Riggs, 1966; Sasaki & Norman, 1966) have shown that the DNA of mammalian chromosomes is arranged in the form of long fibers. Maximum fiber lengths of 500 μ (from HeLa cells) and 1800 μ (from Chinese hamster cells) have been reported by Cairns (1966) and Huberman & Riggs (1966), respectively, for DNA from cells lysed with detergent, while Sasaki & Norman (1966) have found DNA fibers more than 2 cm long from nuclei of human lymphocytes lysed without detergent. In addition, Cairns (1966) has reported the results of pulse experiments which suggest that the long DNA fibers are composed of many separately-replicated, tandemly-joined sections and that the replication process operates at a speed such that the length of newly-replicated DNA in each section increases at a rate of 0.5 μ/min or less. One of the aims of our studies was to verify these findings of Cairns.

Another aim was to determine whether or not the DNA in each of the separate sections is replicated at a fork-like growing point similar to the growing point for E. coli DNA (Cairns, 1963a). Our efforts in this direction met with unexpected success when we found that the separate sections may be replicated at not one, but two, fork-like growing points.

In the remainder of this paper we shall use the word "autoradiogram" to refer to any apparently continuous line of grains presumably

caused by decay of tritium incorporated into DNA, and we shall use the word "fiber" to refer to a single DNA molecule or a single series of DNA molecules joined end-to-end. We shall also refer to the separately-replicated sections of long DNA fibers as "replication units".

2. Materials and Methods

In general the procedures employed were similar to those we have described previously (Huberman & Riggs, 1966). Modifications are given in figure and plate legends.

Incubations with pronase were carried out as described previously (Huberman & Riggs, 1966), except that the DNA was dialyzed against a higher concentration of pronase (1 mg/ml.), and that, after the Millipore filters (Millipore C orp.) were glued to glass slides, they were exposed to a formaldehyde-saturated atmosphere for 36 hrs and then covered with a thin parlodion film.

3. Results

(a) "Cold" pulse labeling experiments

(i) Significance of tandem arrays. When Chinese hamster cells were briefly exposed to ^{3}H-thymidine, then immediately lysed and the released DNA subjected to autoradiography, tandem arrays of DNA autoradiograms,

similar to those reported by Cairns (1966), were sometimes found (Plate I). In order to prove that these tandem arrays were the result of separate replication units in single DNA fibers and not the result of side-by-side aggregation of several separate DNA fibers, each containing a single replication unit, we performed the following experiments.

We reasoned that if all the DNA fibers were completely labeled by growing the cells in high specific activity ^{3}H-thymidine for 24 hrs then side-by-side aggregates could be distinguished by their higher grain density; we also reasoned that if, during the 24 hr period, we exposed the cells to ^{3}H-thymidine of a lower specific activity for a short interval, we could distinguish the autoradiograms of DNA replicated during that short interval by their lower grain density.

If each DNA fiber contained just one replication unit, then after a "cold" pulse experiment of the type described here one would expect to find no more than one low grain density ("cold") region in any long DNA autoradiogram. On the other hand, if each DNA fiber could contain several replication units, then after a "cold" pulse experiment one would expect to find some single long DNA autoradiograms containing several "cold" regions.

The actual labeling sequences used in these experiments are summarized in Figure 1. When the stripping films were exposed for sufficient time to bring out the low specific activity regions (three months or more), long DNA autoradiograms containing several regions of low grain density were frequently found for "cold" pulsed DNA but not for control DNA (Plate II). Thus Cairns' (1966) hypothesis that the

Plate I. Tandem arrays of autoradiograms. Cells of Chinese hamster fibroblast strain B14FAF28 (a gift from Dr. T.C. Hsu) were grown as monolayer cultures on plastic Petri dishes in Eagle's medium supplemented with 10% calf serum. After a 12 hr pretreatment with FUDR (0.1 μg/ml.), ^{3}H-thymidine (18 C/mM, Nuclear Chicago) was added to 0.5 μg/ml. Thirty min later the cells were harvested by trypsinization and diluted to 1×10^{4} cells/ml. in isotonic saline. The solutions used for trypsinization and dilution contained FUDR (0.1 μg/ml.). The cells were diluted ten-fold into "lysis medium" (1.0 M sucrose, 0.05 M NaCl, 0.01 M EDTA, pH 8.0), lysed by dialysis against 1% sodium dodecyl sulfate in "lysis medium", then dialyzed further against "dialysis medium" (0.05 M NaCl, 0.005 M EDTA, pH 8.0). The released DNA was trapped on Millipore VM filters which had served as dialysis membranes. It was then subjected to autoradiography.

Exposure time with Kodak AR-10 Autoradiographic Stripping Film (Eastman Kodak Co.) was 4 months. Picture taken by dark field microscopy.

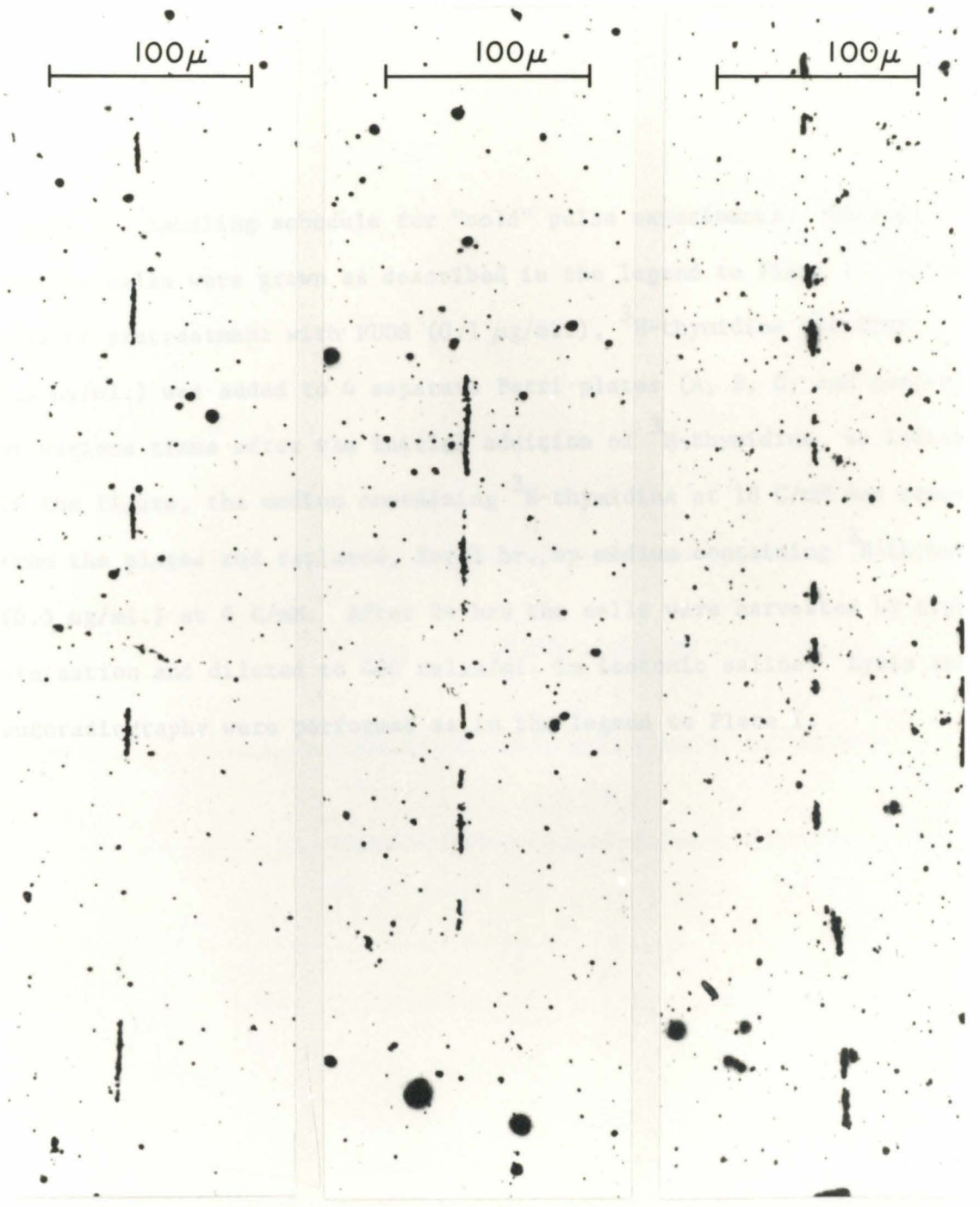

100μ
100μ
100μ
a
b
c

Figure 1. Labeling schedule for "cold" pulse experiments. Chinese hamster cells were grown as described in the legend to Plate I. After a 12 hr pretreatment with FUDR (0.1 μg/ml.), ^{3}H-thymidine (18 C/mM; 0.5 μg/ml.) was added to 4 separate Petri plates (A, B, C, and control). At various times after the initial addition of ^{3}H-thymidine, as indicated in the figure, the medium containing ^{3}H-thymidine at 18 C/mM was removed from the plates and replaced, for 1 hr., by medium containing ^{3}H-thymidine (0.5 μg/ml.) at 6 C/mM. After 24 hrs the cells were harvested by trypsinization and diluted to 400 cells/ml. in isotonic saline. Lysis and autoradiography were performed as in the legend to Plate I.

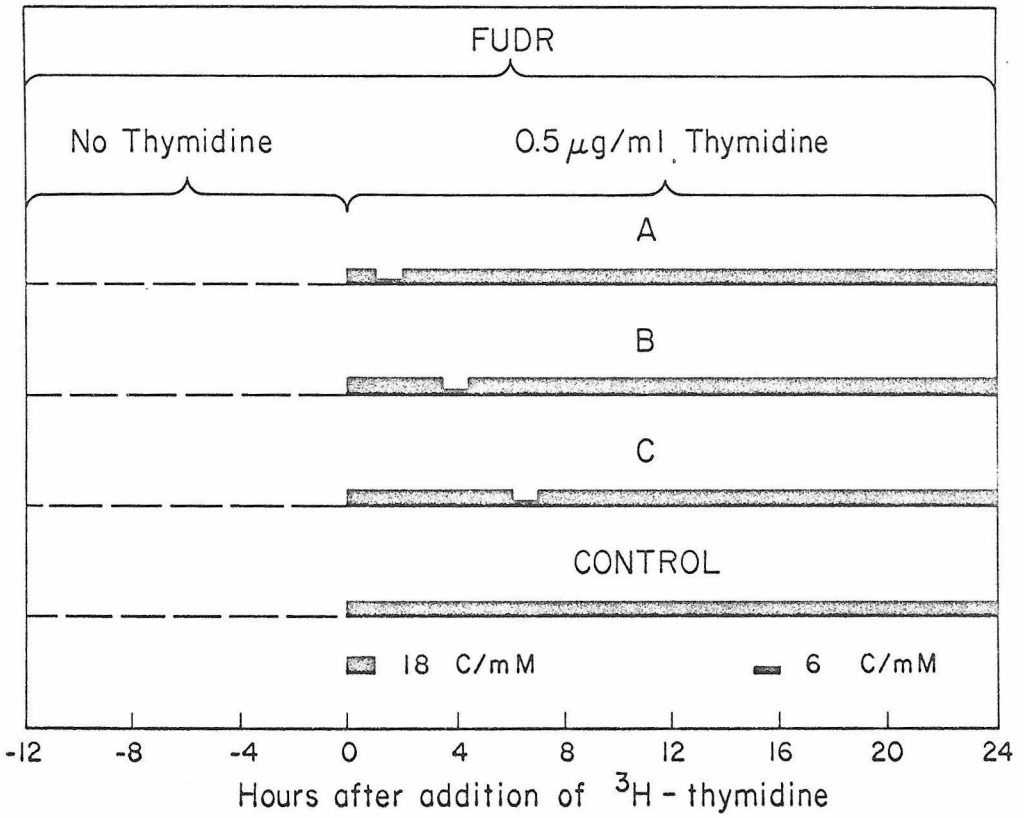
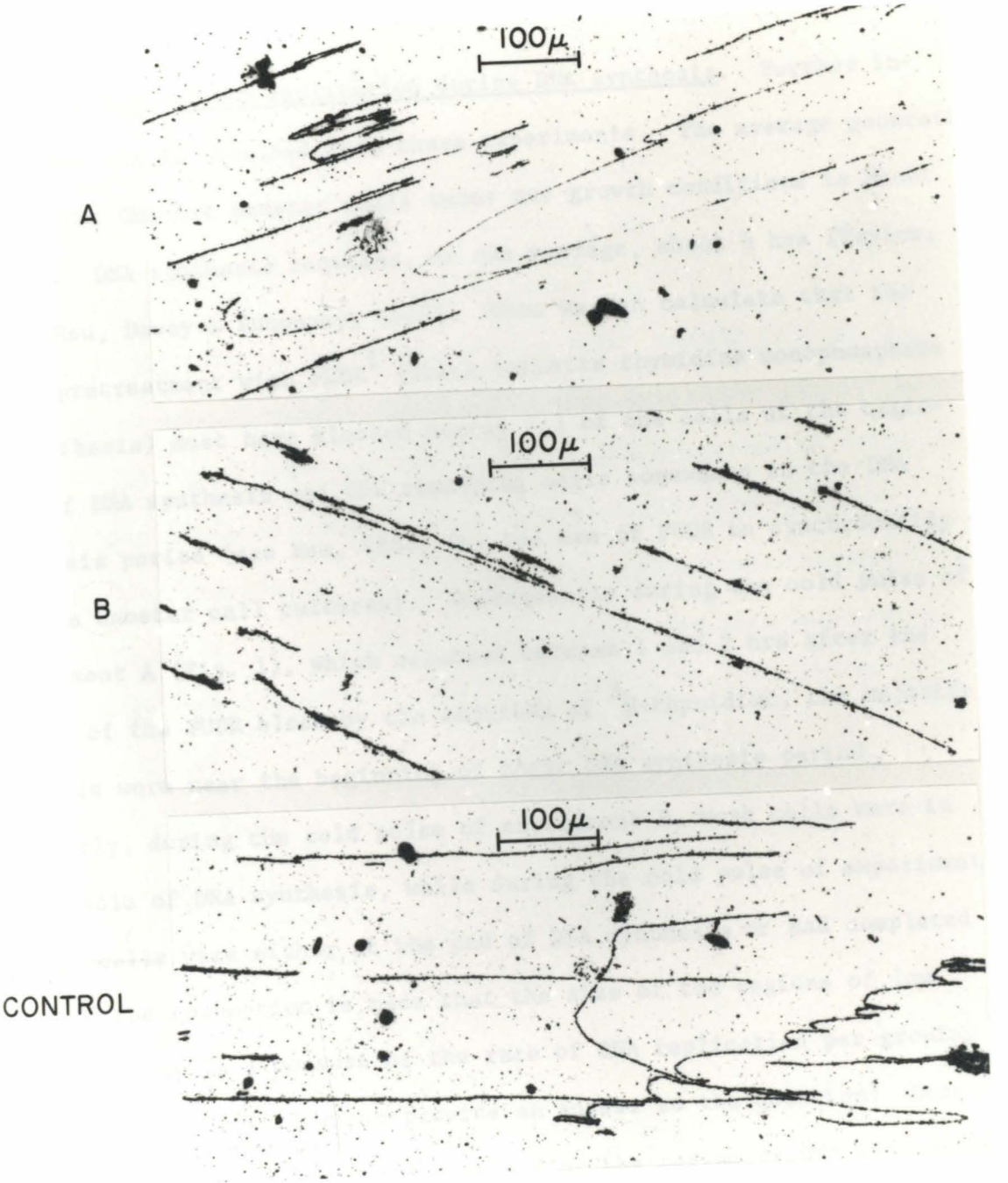

FUDR
No Thymidine
0.5 μg/ml Thymidine
A
B
C
CONTROL
18 C/mM
6 C/mM
-12
-8
-4
0
4
8
12
16
20
24
Hours after addition of ³H - thymidine

Figure 1.

Plate II. "Cold" pulse autoradiograms. Chinese hamster cells were labeled as described in Fig. 1 (A, B and control) and their DNA was subjected to autoradiography. Exposure time was 6 months. Dark field.


100μ
A
100μ
B
100μ
CONTROL

long DNA fibers are composed of many tandemly-joined replication units is proved.

(ii) Rate of DNA replication during DNA synthesis. Further information can be obtained from these experiments. The average generation time of the Chinese hamster cells under our growth conditions is about 17 hrs. DNA synthesis requires, on the average, about 6 hrs (Taylor, 1960; Hsu, Dewey & Humphrey, 1962). Thus we can calculate that the 12 hr pretreatment with FUDR[1] (which inhibits thymidine monophosphate biosynthesis) must have blocked nearly 2/3 of the cells at the beginning of DNA synthesis and the remaining cells somewhere in the DNA synthesis period (see Hsu, 1964, for the use of FUDR in synchronizing Chinese hamster cell cultures). Consequently during the cold pulse of Experiment A (Fig. 1), which occurred between 1 and 2 hrs after the relief of the FUDR block by the addition of ^{3}H-thymidine, the majority of cells were near the beginning of their DNA synthesis period. Similarly, during the cold pulse of experiment B, most cells were in the middle of DNA synthesis, while during the cold pulse of experiment C most cells were either at the end of DNA synthesis or had completed it. If the assumption is made that the size of the regions of low grain density is a measure of the rate of DNA replication per growing point, then our results can provide an answer to the question: Does the rate of DNA replication change during the period of DNA synthesis?

The length distributions of low grain density regions for experiments A, B and C are shown in Figure 2. Low grain density regions less than 20 μ long were not easily resolved. However, for the low

[1]Abbreviations used: FUDR, 5 -flurodeoxyuridine; BUDR, 5-bromodeoxyuridine

Figure 2. Distribution of lengths of low grain density regions in "cold" pulse autoradiograms. Chinese hamster cells were labeled as described in Fig. 1 (A, B and C), and their DNA subjected to autoradiography. The autoradiograms were examined by dark-field microscopy at 100 X. The contours of low grain density regions were traced with a camera lucida, and the lengths of the tracings were then measured and corrected for magnification.

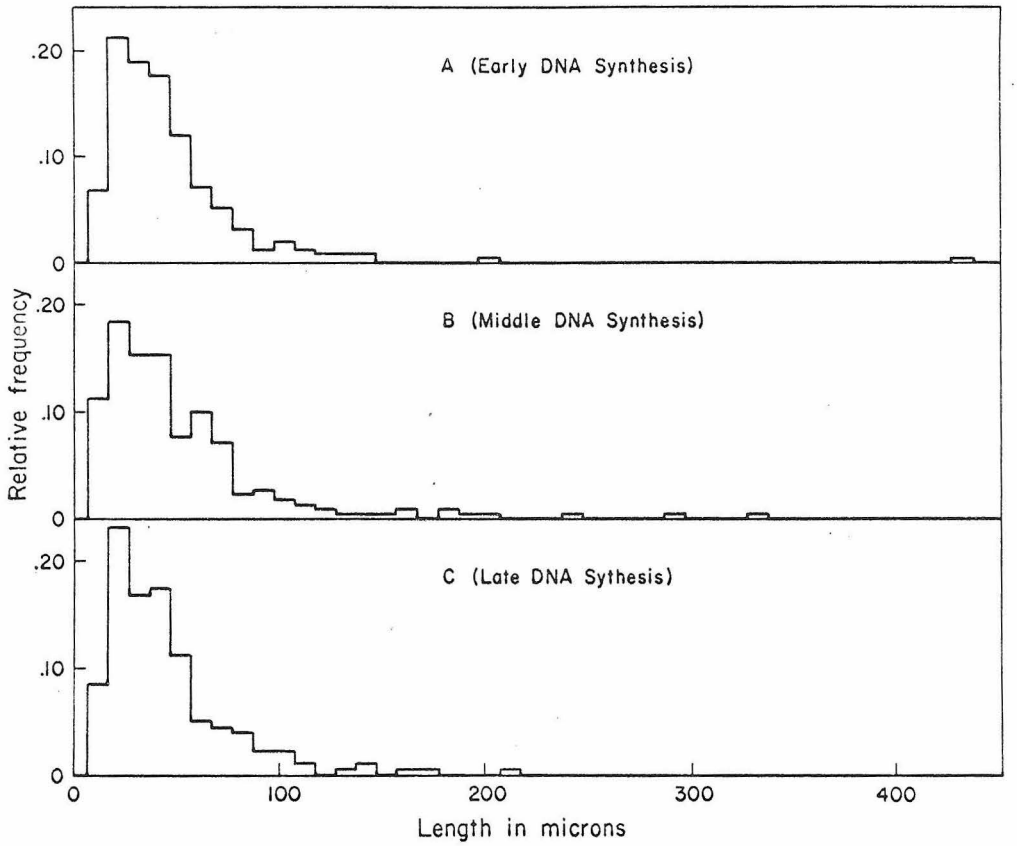

A (Early DNA Synthesis)
B (Middle DNA Synthesis)
C (Late DNA Sythesis)
Relative frequency
.20
.10
0
100
200
300
400
Length in microns

Figure 2.

grain density regions of resolvable size there is no large difference among the three distributions. Thus there is apparently no large change in the rate of DNA replication per growing point during DNA synthesis.

(iii) Distribution of replicating DNA. In "cold" pulse experiments A and B approximately 12% of the total length of autoradiograms was of low grain density; in experiment C regions of low grain density contributed about 7% of the total length. However, in experiments A and B about two-thirds of the autoradiograms contained no regions of low grain density at all; in experiment C four-fifths of the autoradiograms contained no regions of low grain density.

Thus, during any one hour period, regions of DNA synthesis are not distributed equidistantly along the DNA fibers in chromosomes. The nonuniform distribution of regions of DNA synthesis is apparent in Plate II.

(b) Effect of pronase on autoradiogram lengths

We have previously shown (Huberman & Riggs, 1966) that low concentrations of the proteolytic enzyme, pronase, acting on the long DNA fibers in solution prior to autoradiography have no effect on autoradiogram lengths.

However, the finding that each long DNA fiber may contain many separate replication units made imperative an investigation with higher concentrations of pronase. In our earlier experiments we were unable to use higher pronase concentrations because some residual pronase,

adsorbed to the Millipore filters, digested the stripping film. Therefore, in order to prevent this problem, we inactivated the residual pronase with formaldehyde vapors and protected the stripping film with a thin parlodion film placed between it and the Millipore filter. We now report that incubation of the long DNA fibers (from cells exposed to ^{3}H-thymidine for 35 hrs) with about 100 µg/ml. of pronase for up to 6 hrs at 34^{o}C has no effect on either the maximum length or general length distribution of the autoradiograms. Thus any "linkers" connecting the separate replication units, or connecting other points in the long DNA fibers, must be resistant to pronase. Large protein "linkers" are therefore extremely unlikely.

(c) Thirty minute pulse labeling experiments

(i) Does chromosomal DNA replicate at a fork? For complete autoradiographic visualization of a replication fork like that of E. coli (Cairns, 1963a), it is necessary that all three branches of the fork be labeled. This requires that cells complete more than one generation while incorporating ^{3}H-thymidine. We have found, however, that extensive incorporation of ^{3}H-thymidine at specific activities adequate for DNA autoradiography completely prevents division of Chinese hamster cells. Consequently we have been forced to use a less direct method in our attempt to demonstrate the presence or absence of replication forks in Chinese hamster chromosomal DNA.

An appropriate method was suggested by an experiment of Cairns (1963a). He compared the DNA autoradiograms from E. coli cells

exposed to ^{3}H-thymidine for a short time and then immediately lysed (simple pulse) with the DNA autoradiograms from E. coli cells exposed first to ^{3}H-thymidine and then to nonradioactive thymidine and then lysed (pulse-chase). In the case of cells given a simple pulse, the autoradiograms seemed to be the result of two DNA fibers lying side by side; in contrast, the autoradiograms from the cells given a pulse-chase appeared to have been produced by single DNA fibers. Cairns suggested that the different appearance of the two types of autoradiograms was the consequence of replication at a fork-like growing point; the labeled regions of DNA from cells given a simple pulse would be held together at the replication fork and might well appear side-by-side in autoradiograms, while the labeled regions of DNA from cells given a pulse-chase would be separated from the replication fork by a considerable length of unlabeled DNA and would be much more likely to appear separated in autoradiograms. These possibilities are diagramed in Figure 3.

We reasoned that fork-type replication in Chinese hamster cells, if present, should produce the same phenomenon. To test this possibility we performed an analogous experiment with Chinese hamster cells. We used a pulse time of 30 min and a chase time of 45 min. In the case of the simple pulse, all the media to which the cells were exposed after removal from the pulse medium contained FUDR to prevent cellular synthesis of unlabeled thymidine monophosphate.

In some respects our results were different from those Cairns (1963a) obtained with E. coli. For instance, very few of the autoradiograms

Figure 3. The effect of replication at a fork-like growing point on the autoradiograms seen after simple pulse and pulse-chase experiments.

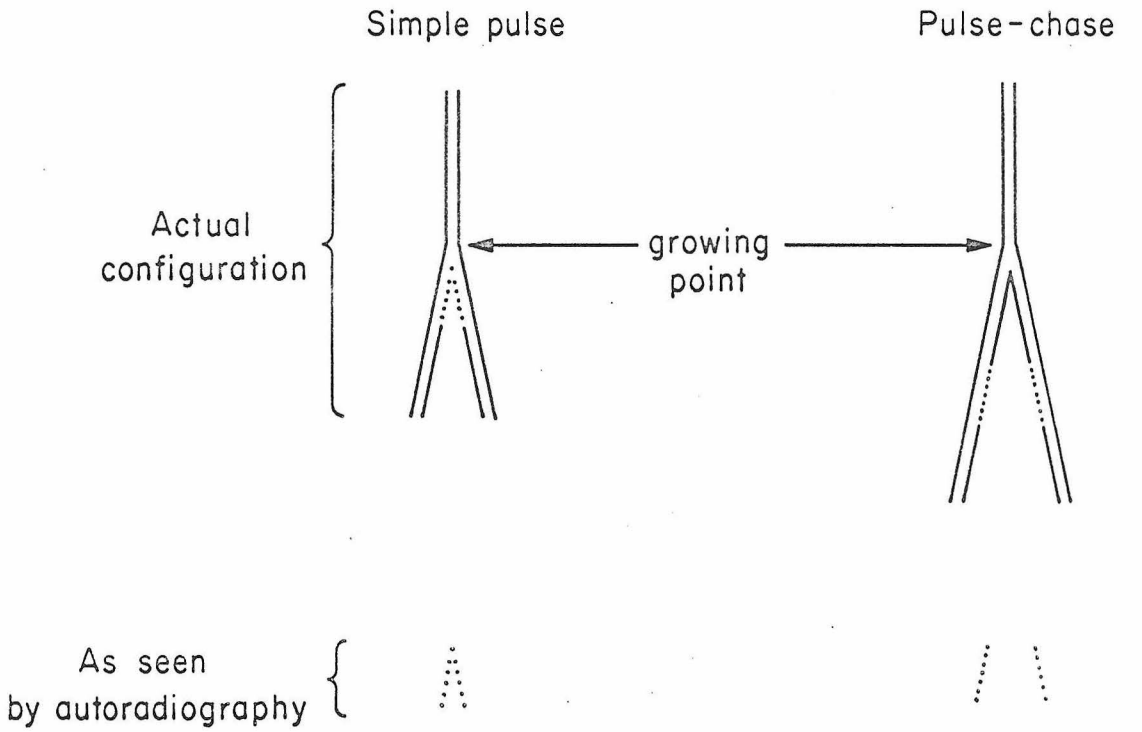

Simple pulse
Pulse-chase
Actual configuration
growing point
As seen by autoradiography

Figure 3.

obtained after simple pulse labeling had an appearance suggesting that they might be the result of two DNA fibers lysing side by side. That is, most such autoradiograms were just single lines of grains with no evidence of separation into two lines of grains. In those few cases where some separation occurred, the separation appeared to be in the middle of the labeled regions rather than at their ends (see below). It is true, however, that many of the autoradiograms obtained after simple pulse labeling had a grain density corresponding to that expected for two DNA double helices lying side-by-side if each double helix were labeled in a single polynucleotide chain. In Figure 4 are shown histograms of grain-density frequency for both the simple pulse autoradiograms and the pulse-chase autoradiograms. Since a grain density of about 2.5 grains/μ is just slightly less (after correction for specific activity, exposure time, and base composition) than the grain density obtained by Cairns (1963b) for a double helix of E. coli DNA labeled in a single polynucleotide chain, each autoradiogram having approximately this grain density was probably produced by a double helix containing a single labeled chain. Likewise, autoradiograms having a grain density of about 5 grains/μ were probably produced by two double helices lying side-by-side and each containing a single labeled chain. The broadness of the distributions can be attributed partly to the statistical error involved in measuring grain density in the shorter autoradiograms and partly to actual errors in grain counting. The higher grain densities may have been underestimated due to the difficulty in distinguishing grains in crowded areas. It is clear, however, that while most of the pulse-chase

Figure 4. Frequencies of grain densities in simple pulse and pulse-chase experiments. Chinese hamster cells were labeled as described in the legend to Plate I (simple pulse) or Plate III (pulse-chase) and their DNA subjected to autoradiography. Exposure time was 4 months. Grain densities were measured with bright field microscopy at 1000 X in all the autoradiograms visible over large areas of single Millipore filters. Measurements were made by counting all the grains of each autoradiogram and dividing by the length of the autoradiogram (determined with an eyepiece micrometer). The total length of all autoradiograms in the sample (more than 100 autoradiograms for both distributions shown) having a given grain density is plotted in the figure as a function of grain density. ————, pulse-chase; -------, simple pulse. The brackets in the figure indicate the grain densities presumed to correspond to 1 or 2 labeled polynucleotide chains.

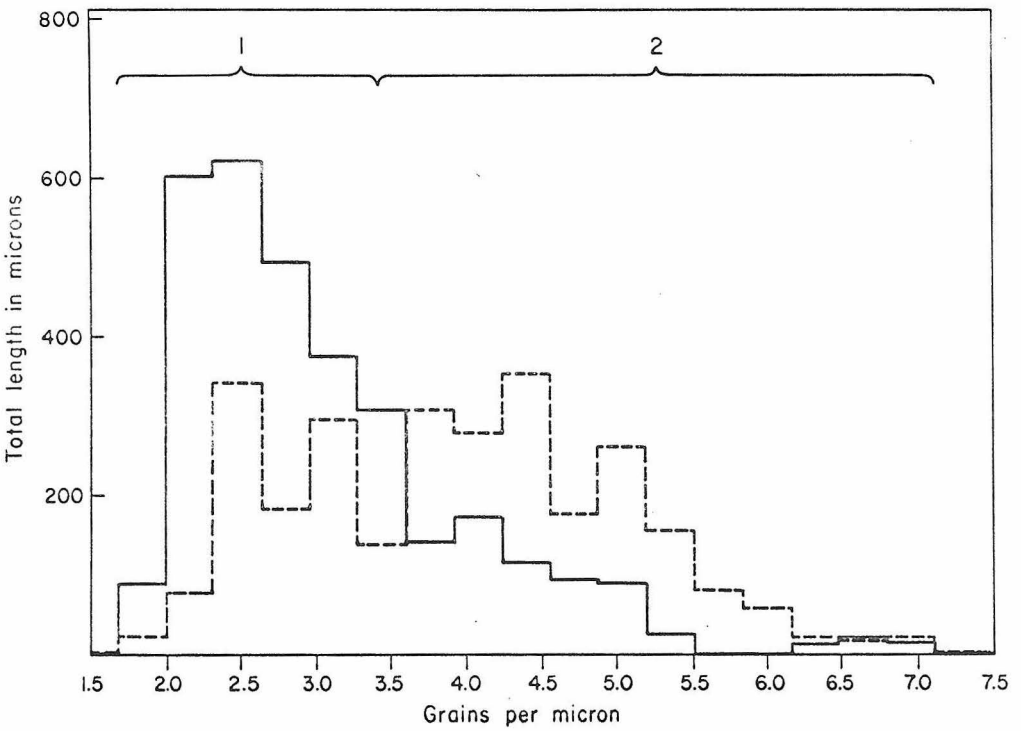

800
600
400
200
Total length in microns
1
2
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Grains per micron

Figure 4.

autoradiograms were produced by single labeled DNA chains (a few pulse-chase autoradiograms produced by two labeled chains would be expected as a result of incomplete chain separation), half or more of the simple pulse autoradiograms were probably produced by two labeled DNA chains (the other simple pulse autoradiograms could be explained as the result of breakage). These results suggest that separation of the chains of the parental double helix takes place during or after replication, rather than before replication, and thus are consistent with replication at a fork-like growing point.

(ii) Grain density gradients. Comparison of typical simple pulse autoradiograms (Plate I) with typical pulse-chase autoradiograms (Plate III) shows another difference besides those mentioned above. Whereas the ends of the simple pulse autoradiograms are always distinct, showing no decline in grain density, the ends of the pulse-chase autoradiograms are frequently indistinct because of a gradual decline in grain density from the full grain density expected for one or two labeled chains of DNA at the center of the autoradiograms to undetectable grain density at the ends.

We interpret this reduction in grain density to be the result of gradual change in the specific activity of the intracellular thymidine triphosphate pool upon replacement of the ^{3}H-thymidine in the external medium with nonradioactive thymidine. Accordingly, for the grain-counting experiments above, we counted grains only in internal regions of the pulse-chase autoradiograms, where no decline in grain density was evident.

Plate III. Typical autoradiograms produced in a pulse-chase experiment. Chinese hamster cells were labeled as described in the legend to Plate I, except that after 30 min of incubation with ^{3}H-thymidine and FUDR, the medium containing ^{3}H-thymidine and FUDR was removed and replaced with medium containing nonradioactive thymidine (5 µg/ml.) and no FUDR. Incubation was continued for 45 min. The cells were then harvested by trypsinization and diluted to 1 x 10^{4} cells/ml. in isotonic saline. Lysis and autoradiography were performed as in the legend to Plate I. Exposure time was 4 months. Dark field.


100 μ

If our interpretation of the grain density gradients is correct, then the direction of decline in grain density is the direction in which DNA was being synthesized at the beginning of the cold chase. Therefore we were surprised to find that many of the autoradiograms had grain densities declining at both ends, in opposite directions (Plate III). In fact, about 35% of the pulse-chase autoradiograms had such gradients at both ends, about 50% had a grain density gradient at one end, and about 15% did not have gradients at either end. The high frequency of double gradients suggests that replication can proceed in both directions in single DNA fibers.

When tandem arrays of pulse-chase autoradiograms were examined, the double gradients proved to be even more prevalent. Nearly all of the internal (and therefore unbroken) autoradiograms in tandem arrays had declining grain densities at both ends (Plate IV). Thus nearly all of the internal autoradiograms in tandem arrays must have been the result of two growing points proceeding in opposite directions.

Exceptions to this rule were very difficult to find. Some examples of apparent exceptions are shown in Plates Va, b and c. The central structure in Plate Va may be an internal autoradiogram without grain density gradients at either end. In Plates Vb and Vc are examples of internal autoradiograms apparently lacking gradients at one end.

Another kind of apparent exception to the rule of double gradients in tandem arrays is shown by the structures in Plates VIc and VIIa. Here the distinct autoradiogram ends are paired opposite each other in adjacent autoradiograms, and there are no grains between the paired

Plate IV. Tandem arrays of autoradiograms produced in a pulse-chase experiment. Labeling and autoradiography were performed as in the legend to Plate III. Dark field.


100μ
100μ
100μ
a
b
c

Plate V. Examples of autoradiograms produced in a pulse-chase experiment. Labeling and autoradiography were performed as in the legend to Plate III.

(a) Example of autoradiogram without grain density gradients at either end. Picture taken by bright field microscopy.

(b) Example of autoradiogram lacking a grain density gradient at one end. Bright field.

(c) Example of autoradiogram lacking a grain density gradient at one end. Dark field.

(d) Example of apparent asynchronous initiation of DNA replication in neighboring replication units. Note that this is also an example of sister double helix separation. Dark field.


25μ
25μ
25μ
100μ
a
b
c
d

Plate VI. Examples of separation of sister double helices. Labeling and autoradiography were performed as in the legend to Plate III.

(a) Bright field.

(b) and (c) Dark field.

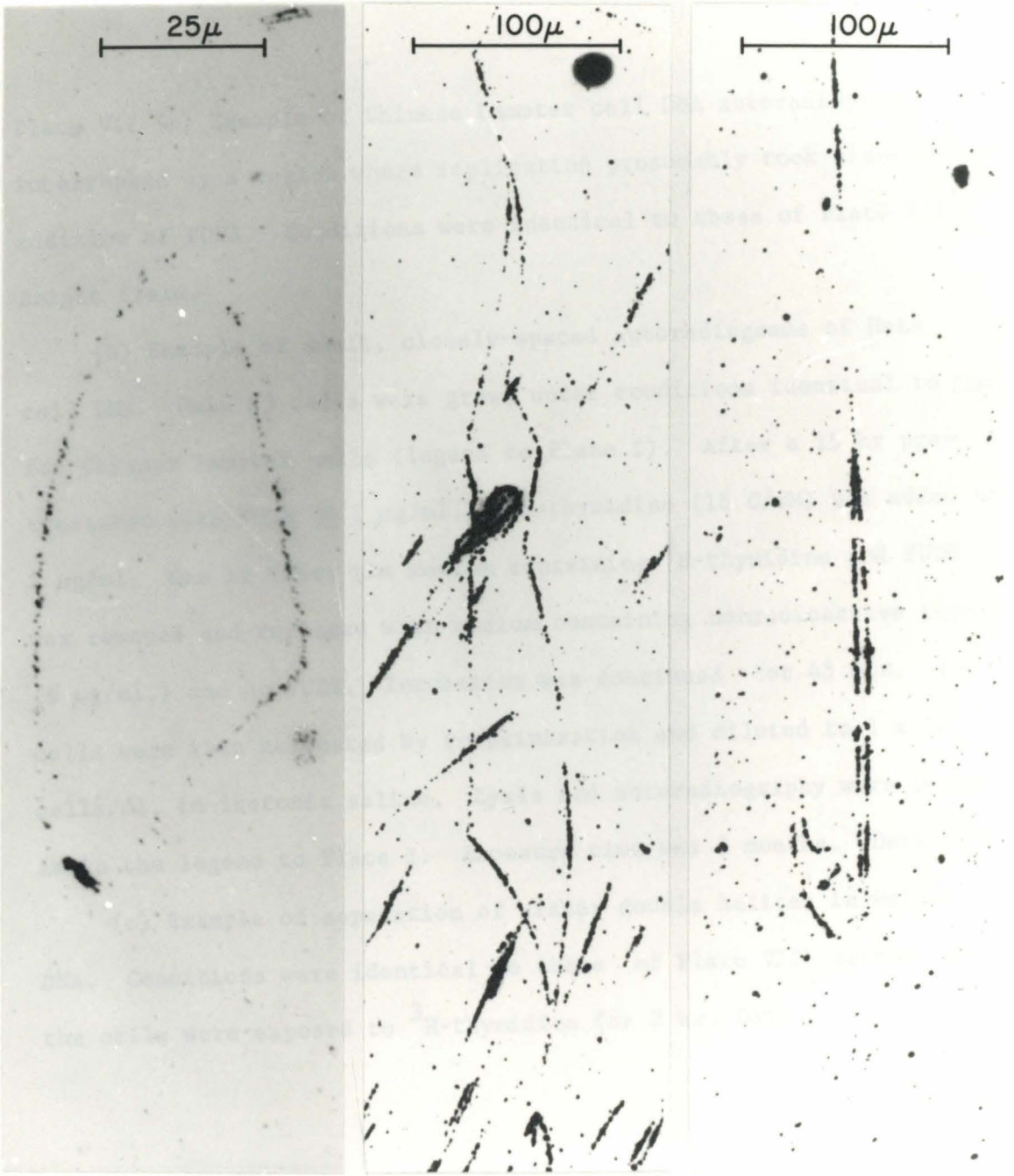
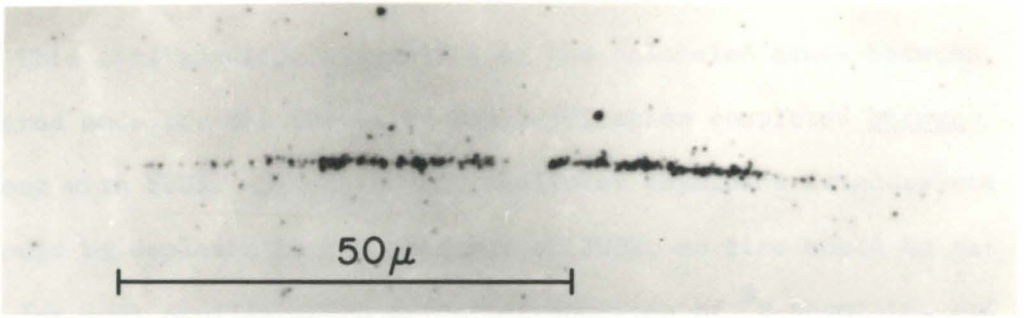

25μ
100μ
100μ
a
b
c

Plate VII (a) Example of Chinese hamster cell DNA autoradiogram interrupted by a region where replication presumably took place before addition of FUDR. Conditions were identical to those of Plate III. Bright field.

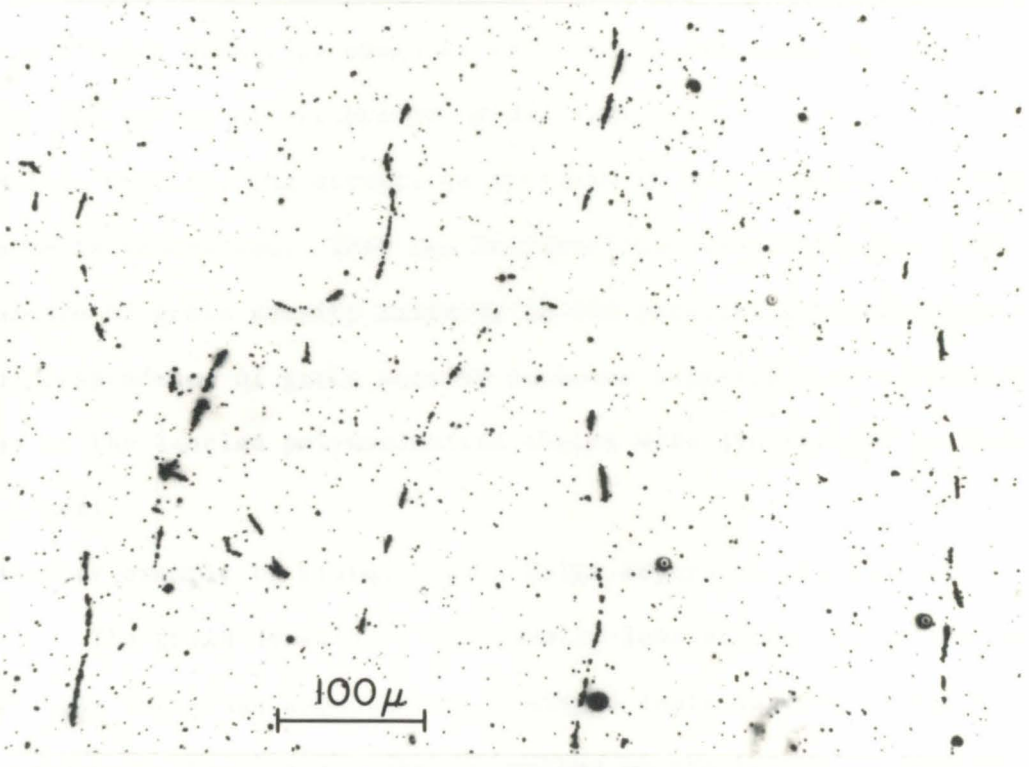
(b) Example of small, closely-spaced autoradiograms of HeLa cell DNA. HeLa S3 cells were grown under conditions identical to those for Chinese hamster cells (legend to Plate I). After a 15 hr pre-treatment with FUDR (0.1 μg/ml.), ^{3}H-thymidine (18 C/mM) was added to 2 μg/ml. One hr later the medium containing ^{3}H-thymidine and FUDR was removed and replaced with medium containing nonradioactive thymidine (5 μg/ml.) and no FUDR. Incubation was continued for 45 min. The cells were then harvested by trypsinization and diluted to 1×10^{4} cells/ml. in isotonic saline. Lysis and autoradiography were performed as in the legend to Plate I. Exposure time was 3 months. Dark field.

(c) Example of separation of sister double helices in HeLa cell DNA. Conditions were identical to those of Plate VIIb except that the cells were exposed to ^{3}H-thymidine for 2 hr. Dark field.

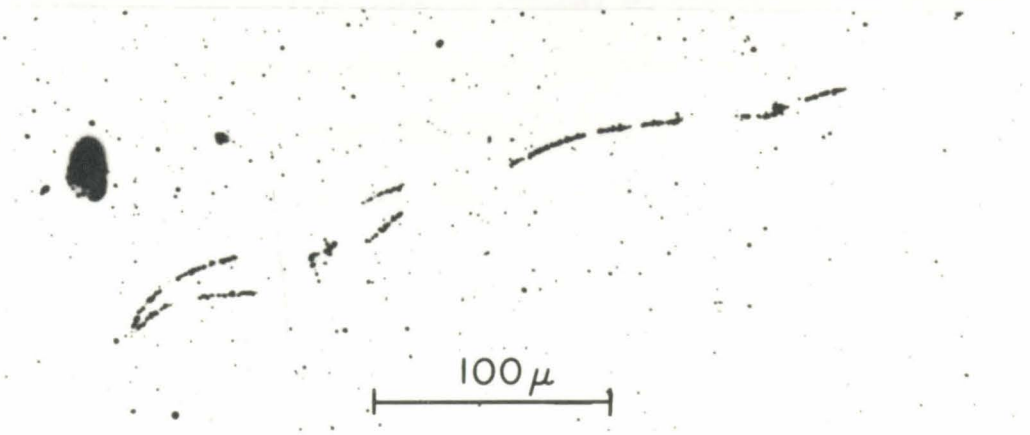
a



b



c



ends. This configuration suggests that the unlabeled areas between the paired ends are the result of DNA replication completed before treatment with FUDR. Since the intracellular thymidine triphosphate pool would be depleted in the presence of FUDR, no time would be required for pool equilibration after introduction of ^{3}H-thymidine and no grain density gradients would be seen in the resulting autoradiograms at the points of resumption of DNA synthesis.

Notice that all the structures in Plate VI are examples of sister double helix separation. They can be identified as such by the correspondence of grain density patterns in the parallel autoradiograms. This correspondence of grain density patterns suggests that corresponding regions of the labeled polynucleotide chains were synthesized at identical times.

Another example of sister double helix separation is shown in Plate Vd. The grain density in the heavily-labeled regions on the top is much less than that expected for a single chain labeled during the pulse (shown in the heavily-labeled regions on the bottom). Thus the heavily-labeled regions on the top may represent points where DNA synthesis was initiated after the pulse, during the period of pool equilibration. If this is true then Plate Vd offers an example of neighboring replication units beginning replication at different times.

(iii) The size of replication units. We have used the tandem arrays of autoradiograms resulting from the thirty minute pulse and pulse-chase treatments to obtain an estimate of the size of the

replication units in Chinese hamster chromosomal DNA. Separate estimates were made for the simple pulse autoradiograms and for the pulse-chase autoradiograms. To avoid errors resulting from possible breaks in DNA fibers at the ends of tandem arrays, only internal autoradiograms of the tandem arrays were used for measurement unless, in the case of pulse-chase autoradiograms, a grain density gradient was present at the end of the array. The method of measurement is diagramed in Figure 5. For the rationale behind this procedure see the Discussion section. Note that the measured quantities were the center-to-center distances between internal or unbroken external autoradiograms of tandem arrays.

Histograms showing the frequency distributions of center-to-center distances are shown in Figure 6. Note that measurements of simple pulse autoradiograms and of pulse-chase autoradiograms gave similar results. The most frequently observed distances are between 15 and 60 μ.

The accuracy of these measurements is not certain. The frequency of distances less than 15 μ may have been underestimated due to difficulties in resolution. In addition, the measurements were based on the assumption that each replication unit in the DNA fibers forming the tandem arrays would be represented by a separate autoradiogram. The possibility that adjacent replication units might have completed replication during the pulse so that their autoradiograms would be fused and the possibility that some replication units might not have begun to replicate during the pulse would thus result in an exaggerated estimate of replication unit size.

Figure 5. Method of measurement of replication unit size from tandem arrays of autoradiograms. The horizontal lines indicate representative autoradiograms. Grain density gradients at the ends of pulse-chase autoradiograms are indicated by dots. The brackets show the lengths which were measured (center-to-center distances between internal or unbroken external autoradiograms).

Simple pulse autoradiograms

Pulse – chase autoradiograms

Figure 5.

Figure 6. Distribution of center-to-center distances between autoradiograms of tandem arrays. Chinese hamster cells were labeled either by the simple pulse method (described in the legend to Plate I) or the pulse-chase method (described in the legend to Plate III), and their DNA subjected to autoradiography. Center-to-center distances between autoradiograms of tandem arrays were measured according to the criteria of Fig. 5. Measurements were made using an eye-piece micrometer and bright-field microscopy at 1000 X.

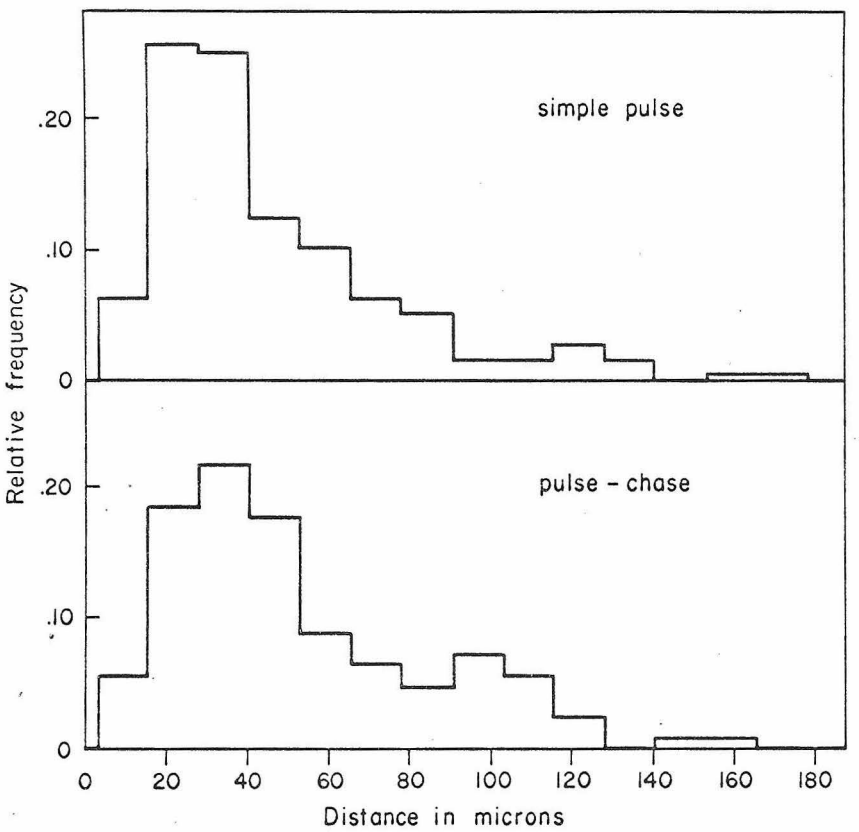

simple pulse
pulse - chase
Relative frequency
.20
.10
0
.20
.10
0
0
20
40
60
80
100
120
140
160
180
Distance in microns

Figure 6.

Since all these uncertainties would cause an overestimate of replication unit size, one can conclude that most replication units are at least as small as indicated by the histograms of Figure 6.

(d) Estimation of replication rate

(i) Estimates from the 30 minute pulse-chase experiment. We shall use the term "replication rate" to refer to the rate at which DNA is synthesized at an individual growing point. This rate will be measured in units of μ of movement of the growing point along the parental DNA double helix per minute (μ/min).

The possibility of autoradiogram fusion discussed above and uncertainties in the time of stopping and starting of replication in individual replication units make estimates of replication rate based on the lengths of autoradiograms produced after a 30 minute pulse extremely uncertain. However, the pulse-chase autoradiograms have provided two alternative methods for estimation of replication rate. First, in most cases the grain densities at the ends of the pulse-chase autoradiograms appear to decrease monotonically, suggesting that, within the gradient region, replication was proceeding at a single growing point throughout the equilibration period. Monotonic gradients 20-40 μ long were frequently found (Plate III) while monotonic gradients as much as 50-100 μ long were occasionally found (Plate IVc). Since the maximum possible equilibration time is 45 min (chase time), a minimum estimate of replication rate is 0.4-0.9 μ/min for the frequently observed gradients and 1-2 μ/min for the occasionally observed gradients. The variations

found in lengths of monotonic gradients can be explained as the result of heterogeneity between cells in pool equilibration time or as the result of heterogeneity of replication rate or both.

In addition, if our interpretation of the sharp interruptions in the autoradiograms shown in Plates VIc and VIIa is correct, then the ends without grain density gradients represent points where replication started at the beginning of the 30-minute pulse. Likewise the positions where the grain density begins to decline in such autoradiograms represent the points to which replication had proceeded by the end of the pulse. We conclude that each branch of such autoradiograms represents a region synthesized during the entire pulse. We have found samples of such autoradiograms with branches from 15 to 37 μ long. On the assumption that each branch is the result of a single growing point, these lengths correspond to replication rates of 0.5-1.2 μ/min.

(ii) The maximum possible replication rate. To measure the maximum possible rate of DNA replication, Chinese hamster cells were exposed to ^{3}H-thymidine for pulse times of 5, 10, 20, 60 and 120 min and their DNA subjected to autoradiography. The maximum autoradiogram lengths found after these pulse times increased more or less linearly with pulse time at a rate of 5-10 μ/min.

These measurements set an upper limit to the replication rate of Chinese hamster DNA. Assuming that each autoradiogram is the product of only two growing points, then the maximum possible rate of replication is 2½ - 5 μ/min. Although the maximum autoradiogram lengths found after the longer pulse times are probably the result of autoradiogram

fusion, the fact that autoradiograms 25 μ long are found even after the shortest pulse time of 5 min suggests that Chinese hamster cell DNA may, indeed, occasionally replicate as rapidly as $2\frac{1}{2}$ μ/min.

(e) Experiments with HeLa cells

In order to compare DNA replication in HeLa cells with that in Chinese hamster cells, we conducted a series of experiments in which we exposed HeLa cells and Chinese hamster cells to ^{3}H-thymidine for times of 1, 2, 4, 8 and 18 hrs. For all these experiments the pulse with ^{3}H-thymidine was followed by a 45 min chase with nonradioactive thymidine. Because we also wanted to investigate the possible effect of FUDR on DNA replication in these cells, some of the experiments were carried out in the absence of FUDR.

In general, the HeLa cell autoradiograms and the Chinese hamster cell autoradiograms were similar in size and appearance. This was especially true for pulse times of 4 hrs or longer. For pulse times of 1 or 2 hrs, however, some differences were evident. First, although the HeLa cells were subjected to the same kind of pulse-chase treatment as the Chinese hamster cells, grain density gradients were difficult to observe in the HeLa cell autoradiograms. What might have been short grain density gradients were found only occasionally. We should point out that even for the Chinese hamster cells grain density gradients were not nearly so frequent in these longer experiments as they were in the 30 minute pulse-chase experiments. This low frequency of gradients has not been investigated further.

Also, although the absence or presence of FUDR had no detectable effect on the size or type of autoradiograms produced by Chinese hamster DNA, the DNA from HeLa cells exposed to FUDR for 15 hrs and then pulse-labeled for 1 hr produced a much greater proportion of very short autoradiograms, usually close together in tandem arrays, than did DNA from Chinese hamster cells or from HeLa cells not exposed to FUDR. An example of these short HeLa cell autoradiograms is shown in Plate VIIb.

The fact that FUDR did not alter the appearance of Chinese hamster cell autoradiograms suggests that it has no effect on DNA autoradiography other than the intended ones of preventing thymidine monophosphate biosynthesis and blocking most of the cells at the beginning of DNA synthesis. Our finding that DNA from HeLa cells treated with FUDR for 15 hrs and then pulse-labeled for 1 hr produces a high proportion of small, close autoradiograms suggests that the DNA which replicates earliest in HeLa cells may be unusually rich in small replication units.

Like the Chinese hamster DNA, the HeLa DNA produced autoradiograms suggesting the separation of sister double helices. Three examples are shown in Plates VIIc and VIII.

4. Discussion

(a) Summary of results

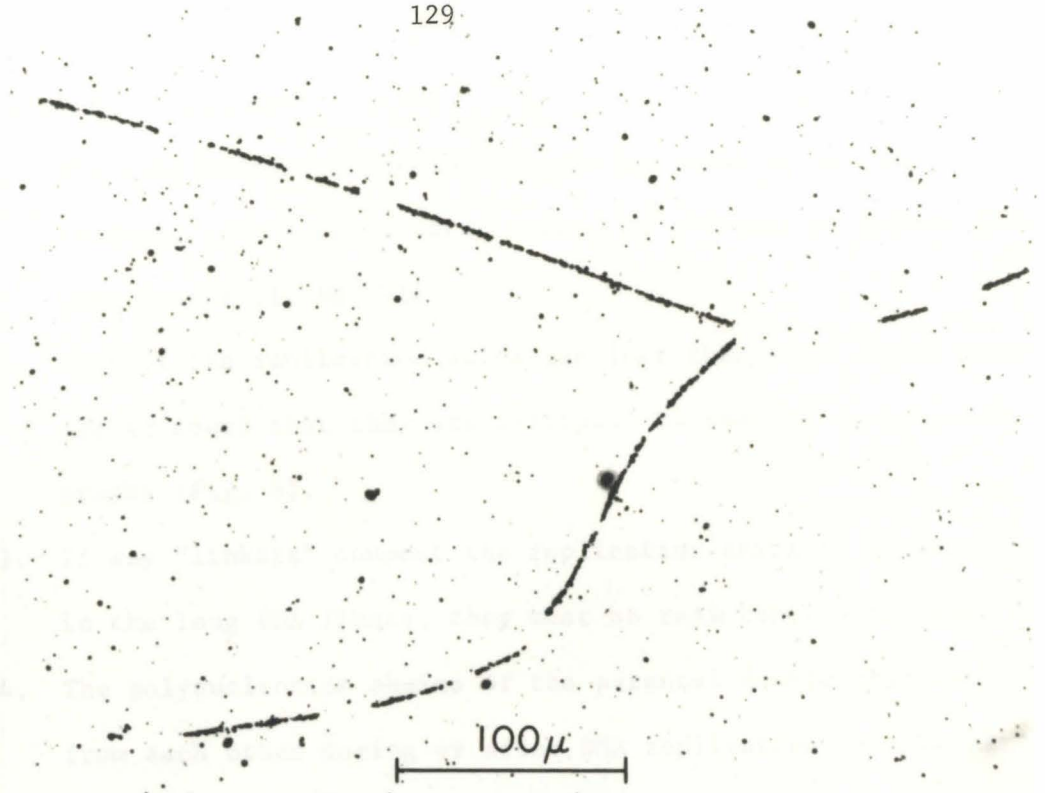
The autoradiographic evidence we have presented above suggests that the process of DNA replication in Chinese hamster chromosomes has

Plate VIII. Examples of separation of sister double helices in HeLa cell DNA.

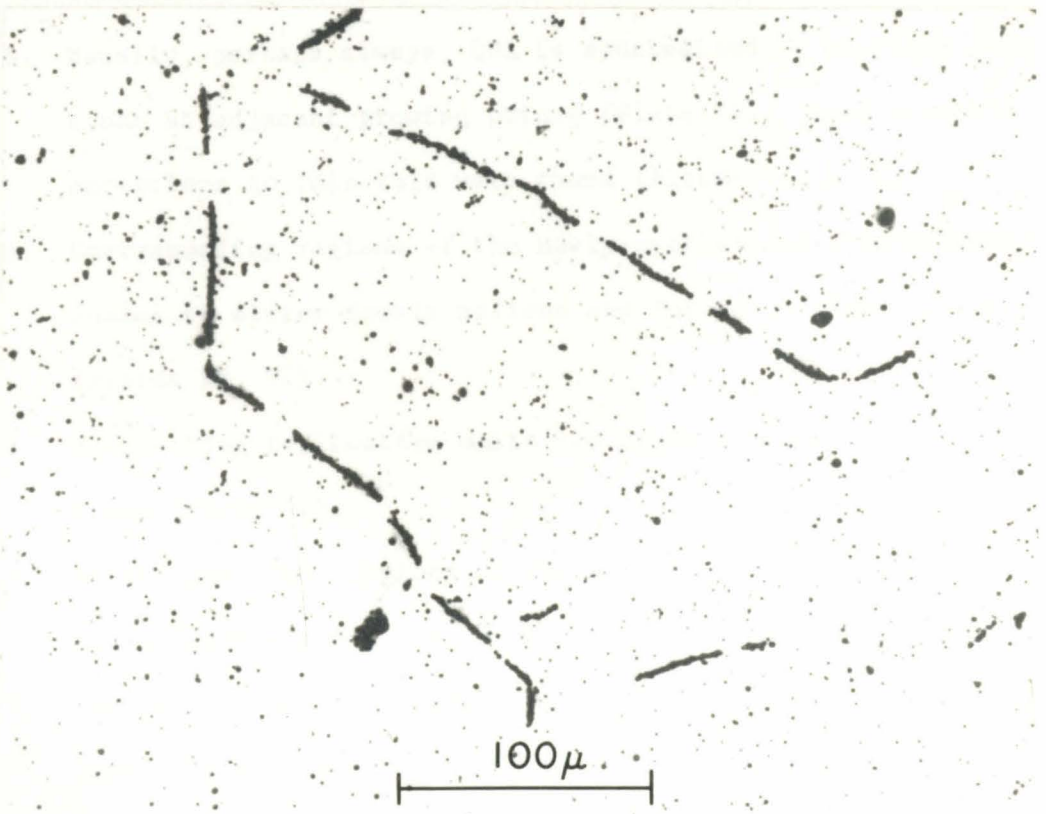
(a) Conditions identical to those of Plate VIIc. Dark field.

(b) Conditions identical to those of Plate VIIb except that there was no pretreatment with FUDR and FUDR was not present during the pulse. Exposure time was 7½ months. Dark field.

a



b



the following attributes:

1. The long fibers of chromosomal DNA are made up of many tamdemly-joined replication units, as proposed by Cairns (1966). (See Plates II, Vd, VI).
2. Most of the replication units are less than 60 μ long. Many are so short that they are difficult to resolve by autoradiography (Fig. 6).
3. If any "linkers" connect the replication units or other points in the long DNA fibers, they must be resistant to pronase.
4. The polynucleotide chains of the parental double helix separate from each other during or after DNA replication in a manner consistent with replication at a fork-like growing point (Fig.4).
5. Usually, perhaps always, DNA is synthesized in opposite directions at adjacent growing points (Plate IV). Rare possible exceptions to this rule were found (Plates Va, b, c).
6. Corresponding regions of the newly-synthesized polynucleotide chains in sister double helices are formed at identical times (Plates Vd, VI).
7. Neighboring replication units can begin replication at different times (Plate Vd).
8. At any one time, regions of DNA synthesis are not distributed equidistantly along the chromosomal DNA fibers (Plate II).
9. The rate of DNA replication per growing point is 2.5 μ/min or less. This rate does not vary greatly during the period of DNA synthesis (Fig. 2).

(b) The bidirectional model

The properties listed above suggest a model for chromosomal DNA replication which we shall call the "bidirectional" model. According to this model, which is diagramed in Figure 7, each replication unit would contain a single origin for DNA replication (marked O) in its interior, and would be separated from adjacent replication units by two termini (marked T). Replication units would be arranged in tandem, terminus-to-terminus, to produce long DNA fibers (Fig. 7a). Within each unit, replication would start at the origin and proceed outwards at two fork-like growing points to the termini, where it would stop (Fig. 7b,c). After adjacent replication units had completed replication, separation of sister double helices could occur (Fig. 7d). Separation of sister double helices might also occur within individual replication units before they had completed replication (Fig. 7b,c).

We shall now consider in detail the evidence for the various aspects of the bidirectional model. A central feature of the model is the concept that replication proceeds at fork-like growing points. The best evidence for the fork-like nature of the growing points is the correspondence of grain density patterns in separated sister double helices. This correspondence is strikingly demonstrated by HeLa cell DNA as well as by Chinese hamster DNA. It means that corresponding regions of the newly-synthesized polynucleotide chains in the sister double helices must have been formed at the same time. Thus it rules out the possibility that, within a given replication unit, DNA could be synthesized first on one

Figure 7. Summary of the bidirectional model for DNA replication. Each pair of horizontal lines represents a section of a double helical DNA molecule containing two polynucleotide chains (———, parental chain; ----, newly synthesized chain). The short vertical lines represent positions of origins (O) and termini (T). The diagrams represent different stages in the replication of two adjacent replication units:

(a) prior to replication

(b) replication started in right-hand unit

(c) replication started in left-hand unit and completed at termini of right-hand unit

(d) replication completed in both units; sister double helices separated at the common terminus

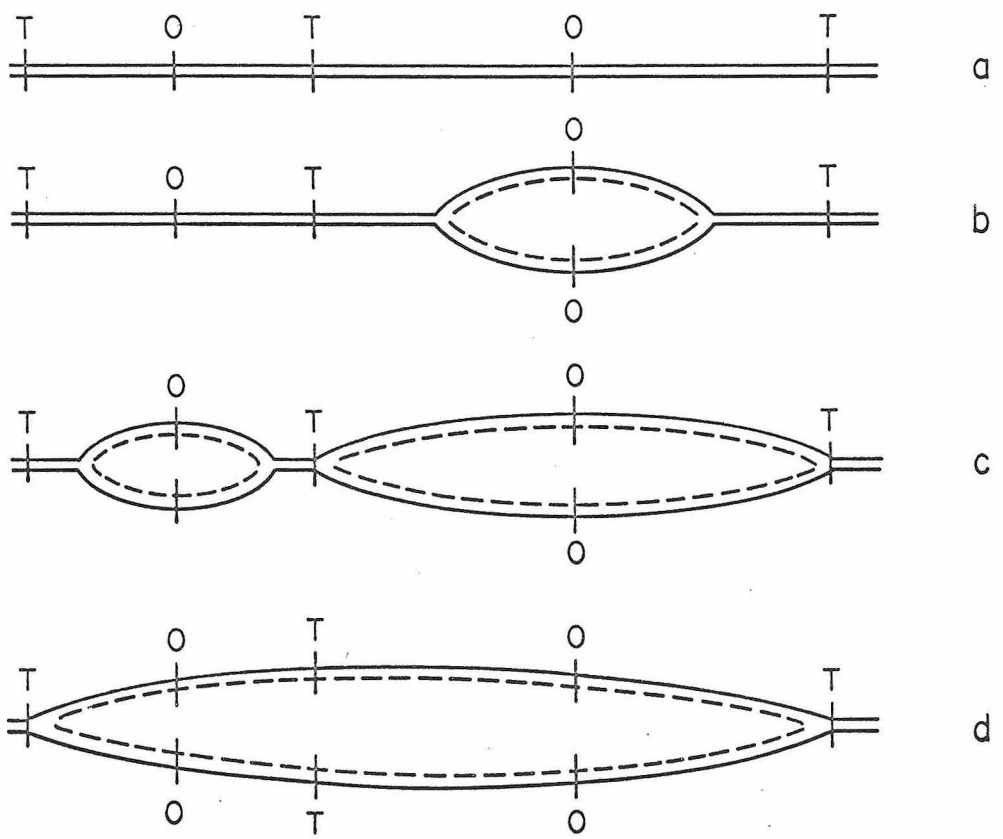


Figure 7.

parental chain as a template and then on the other; within regions of the double helix smaller than thelimit of autoradiographic resolution (about 5 μ) both chains must act as a template at once. Fork-like growing points provide by far the simplest explanation of this behavior. Additional evidence for fork-like growing points is, of course, provided by our finding that complete separation of the parental chains apparently takes place during or after, rather than before, replication.

Another central feature of the bidirectional model is the concept that each replication unit contains two growing points which begin replication at the same time. How can this situation be distinguished from the one where each growing point is under separate control?

It is difficult, with the available evidence, to distinguish conclusively between these alternatives. However, the evidence favors the simultaneous starting of paired growing points. First, the grain density gradients observed in the 30 minute pulse-chase experiment strongly suggest that adjacent growing points proceed in opposite directions. This means that each growing point must share its origin with a neighboring growing point. Synchronous starting of the two growing points at their common origin would be more economical for the cell in terms of the complexity of regulation of DNA synthesis.

Second, the fact that in the 30 minute pulse-chase experiment internal autoradiograms with grain density gradients at only one end were extremely rare (except for those which could be explained by partial replication before addition of FUDR) shows that in most cases, if not all, both growing points at each origin started within the time span of the pulse

plus the period of pool equilibration. Finally, in all structures like those in Plates VIc and VIIa, the regions of interruption where replication presumably took place before the addition of FUDR were enclosed on each side by autoradiogram branches of approximately equal length. The equal branch lengths on each side of the interruption strongly suggest that, once the FUDR block was relieved by ^{3}H-thymidine, both growing points started at the same time.

The bidirectional model also suggests that replication units are separated from each other by structures of unspecified nature called termini, and that replication stops at these termini. An alternative possibility is that there are no structural boundaries between replication units and that the growing points in adjacent replication units continue to operate until they meet each other somewhere between their origins. It is extremely difficult to distinguish between these possibilities. On the one hand, the rarity in the 30 minute pulse-chase experiment of autoradiograms in tandem arrays lacking grain density gradients at either or both ends suggests that few, if any, replication units stop replication before their growing points have met the growing points from adjacent replication units. On the other hand, the few apparent exceptions (Plates Va, b, c) do suggest asynchronous termination of replication by adjacent growing points (the autoradiograms with grain density gradients at only one end in Plates Vb and c might also be explained as exceptions to the rule that adjacent growing points proceed in opposite directions). Thus the proposed termini in the bidirectional model must be considered tentative.

Other features of the bidirectional model such as the tandem arrangement of replication units and the time of separation of sister double helices seem well established by our autoradiographic evidence (Plates II, Vd, VI, VIIc, VIII).

The bidirectional model is obviously incomplete. Some features of the model, especially the termini, are still tentative. Other possible features have not even been suggested as part of the model because of the paucity of available evidence.

At least one swivel point must be present ahead of each growing point during actual replication to allow unwinding of the parental polynucleotide chains. The nature and position of these swivel points are not yet known.

The swivel points might be single-chain breaks. Certainly single-chain breaks must exist temporarily in the newly-synthesized polynucleotide chains between the products of different growing points. The time at which such breaks are healed, and the manner of their healing, are still unknown.

Although the bidirectional model, as presented here, does not require any non-nucleotide linkers in the chains of DNA, the presence of such linkers (if they are pronase-resistant) is certainly possible. In addition other accessory structures may be required during DNA replication. The nature of these hypothetical accessory structures and their manner of duplication are, of course, not known.

It is now well established that, at least at the level of resolution of whole chromosome autoradiography, the timing of replication in the different replication units of eukaryotic chromosomal DNA is exactly and heritably controlled (see Huberman, 1967, for review). Some pulse-chase autoradiograms, like those in Plate Vd, suggest that neighboring replication units can start to replicate at different times, thus supporting the concept that individual replication units can be independently controlled. On the other hand, some of the tandem arrays we have observed (Plates Ia, Ic, IVa, IVb), where the autoradiograms are of approximately uniform length, suggest that common control of neighboring units is likely in some cases. The actual mechanisms by which initiation and termination of replication are controlled in individual replication units are still unknown.

(c) The rate of DNA replication

(i) Comparison of results. The rate of DNA replication in the chromosomes of mammalian cells has previously been measured in two ways. Cairns (1966 and personal communication) measured the lengths of the autoradiograms produced after exposure of HeLa cells to ^{3}H-thymidine for 45 min and 180 min, and concluded that the average length increased at a rate of 0.5 μ/min or less. Taylor (personal communication) measured the BUDR pulse time required before Chinese hamster DNA molecules were fully converted to hybrid density, and estimated from his results that the BUDR-labeled region increased in length at a rate of 1 - 2 μ/min. According to the bidirectional model, these measurements should be

considered overestimates of the actual rate of DNA replication per growing point, because some of the measured lengths, if not most, probably were the result of two growing points.

These measurements are in fairly good agreement with the results we have obtained by a variety of methods. Measurements of the maximum autoradiogram lengths obtained after short pulses suggest that the rate of DNA replication must be less than about 2.5 μ/min. Measurements of the length of DNA synthesized during equilibration of the thymidine triphosphate pool suggest rates of replication from 0.4 to 2 μ/min.

Probably the most reliable estimates are provided by measurements of the lengths of the branches in autoradiograms such as those in Plates VIc and VIIa where it is reasonably certain that DNA synthesis was going on throughout the 30 minute pulse. Such measurements suggest replication rates from 0.5 to 1.2 μ/min.

The uncertainties involved in all these measurements mean that a precise estimate of the replication rate in Chinese hamster cells is still impossible. However, one can draw some general conclusions from the measurements made so far.

All the measurements suggest that there is probably no one replication rate but rather a distribution of replication rates. Whether or not the replication rate in a single replication unit is always constant is not known.

Also, it is interesting that, despite the fact that mammalian replication units are much smaller than bacterial chromosomes, the rate of bacterial DNA replication is much larger (30 μ/min as measured by

Cairns, 1963a) than the rate of mammalian DNA replication. Perhaps the necessity for synthesizing and organizing the many chromosomal proteins of mammalian cells requires a lower rate of replication.

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FURTHER DISCUSSION

(a) The number of DNA fibers per chromatid

One of the still unsolved questions of chromosome structure is the number of DNA fibers per chromatid (I use the word "fiber", again to mean a single DNA molecule or a single series of DNA molecules joined end-to-end). The arguments in favor of a single DNA fiber per chromatid include the apparent analogy, both in segregation (5) and in polarity (6), between the chromatid subunits discovered by Taylor, Woods & Hughes (5) and single polynucleotide chains of DNA. In addition, Gall (7) has found that the loops and axes of lampbrush chromosomes, which consist of one and two chromatids, respectively, are cleaved by pancreatic DNase at rates which are only consistent with the presence of one DNA fiber in each chromatid. Miller (8) has supported the deduction of Gall by showing, with the electron microscope, that the loops and axes of lampbrush chromosomes have the minimum lateral dimensions expected for one and two DNA molecules, respectively.

The arguments in favor of two or more fibers per chromatid are based on the double appearance of chromatids, under some conditions, in the light microscope and on occasional apparent exceptions to the rule of semiconservative segregation of chromatid subunits. These experiments have recently been reviewed by Peacock (9) and Gay (10).

Our earlier autoradiographic results (Chapter I) did not contribute to the resolution of this question. However, Sasaki & Norman (4), using an autoradiographic technique which did not require detergent, have reported finding DNA fibers from human chromosomes over 2 cm long. As this is the total length of DNA expected in a small human chromatid or in the arm of a large chromatid, the results of Sasaki & Norman suggest that the number of DNA fibers per chromatid may be very small, perhaps one or two.

A problem in interpreting the results of Sasaki and Norman, however, has been that many of the very long DNA fibers they found were apparently circular (none of the smaller ones were). Genetically, eukaryotic chromosomes are linear structures. Thus, although very small circular DNA fibers might have been expected in such chromosomes (11), circles as long as 1 or 2 cm are not expected and must be considered suspect.

The bidirectional model suggests a possible explanation for such long circular fibers. As pointed out above, extensive incorporation of ^{3}H-thymidine prevents cell division. The exact stage at which division is inhibited is not known, but it is possible that the tritium could, indirectly, prevent final completion of strand separation in some chromosomes. With the added assumption that final strand separation

occurs at the ends of chromatids, or at the ends and centromeres, the long circular fibers could be explained as sister linear DNA fibers not yet separated at their ends. Certainly, incomplete strand separation over shorter regions produces circle-like structures (Plates VI and VIIc above). This explanation could be tested by a "cold" pulse experiment. If the circular fibers are indeed two sister linear fibers joined at their ends, then it should be possible to divide each circular fiber found after a "cold" pulse experiment into two halves containing identical patterns of "hot" and "cold" regions.

(b) The size of the DNA molecules in chromosomes

Another unsolved problem of chromosome structure is the number of DNA molecules per fiber. The difference between the fiber lengths obtained by Cairns (3) and us (Chapter I) on the one hand, and by Sasaki & Norman (4) on the other, suggests the possibility that some detergent-sensitive "linkers" may exist in the long DNA fibers. The difference can also be explained, of course, by the fact that detergent treatment would probably remove most protein from the DNA fibers so that they would be much more sensitive to shear or nuclease degradation.

Our experiments with pronase (Chapters I and II) show that if linkers exist within Chinese hamster DNA fibers at intervals of less than 1800 μ they must be resistant to pronase. In addition, Macgregor & Callan (12) have tested several proteolytic enzymes, RNase, and DNase for their ability to degrade lampbrush chromosomes. Although the proteolytic enzymes and RNase (all at 250 μg/ml for up to 2 hrs at room temperature)

removed matrix material from the chromosomes, only DNase (at concentrations as low as 12.5 μg/ml) was able to produce actual breaks in the loops or axes of the chromosomes (after just a few minutes at room temperature). Thus the DNA fibers of lampbrush chromosomes apparently contain no linkers sensitive either to proteolytic enzymes or to RNase.

These experiments do not exclude all possible types of linkers, however. Indeed, Bendich & Rosenkranz (13) have proposed that natural DNA may contain linkers of serine or serine-containing oligopeptides about every 1000 nucleotides. Their proposal is based on the findings that DNA purified from many sources still contains small quantities of amino acids (including enough serine to provide one serine for every 500-1000 nucleotides) and that extended treatment with hydroxylamine results in breakage of DNA (from human leukocytes) into fragments with a molecular weight of about 500,000 (or almost 1000 nucleotide pairs). This molecular weight corresponds to a DNA length of about 0.25 μ — considerably smaller than our estimates of replication unit size for Chinese hamster DNA. However, since our estimates only set an upper limit to replication unit size, we cannot rule out the possibility that such linkers, if they exist, are involved in control of DNA replication.

The possibility that replication units may have indefinite boundaries

The studies which have suggested that the timing of replication in the different replication units of chromosomal DNA is heritably controlled (14-42) were all performed at the level of whole chromosome autoradiography. At this level individual replication units are probably not

resolved. It is still possible, therefore, that the boundaries between replication units may not be the same from one generation to the next. That is, neither the previous studies (14-42) nor our present results exclude the possibility that the initiation of replication may be controlled only for large sections of DNA fibers containing many replication units and that within these large sections sites of initiation may be randomly chosen. As noted in the publication section of Chapter II, it is also possible that sites of termination may be indefinite.

Relation to bacterial DNA replication

The available evidence (43-47) suggests that replication of bacterial chromosomes is unidirectional and that normally only one growing point is involved. Nevertheless, a phenomenon observed by Cairns (43) suggests a possible relationship to mammalian DNA replication. One of the autoradiograms of replicating E. coli DNA published by Cairns (Plate IIa in reference 43) shows what appear to be two bacterial chromosomes joined together at their origins and replicating at two growing points (one for each chromosome) away from their origins. Cairns pointed out the possibility that the apparent union of the two chromosomes might have occurred after cell lysis. It may also be possible, though, that in binucleate bacteria the two origins for replication are normally joined and replication thus resembles the bidirectional replication of mammalian DNA. Indeed, it is possible that what we have called a mammalian replication unit is evolutionarily related to two bacterial chromosomes, joined origin-to-origin.

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