AUTORADIOGRAPHY OF CHROMOSOMAL DNA FIBERS FROM CHINESE HAMSTER CELLS*

By Joel A. Huberman and Arthur D. Riggs

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

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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-

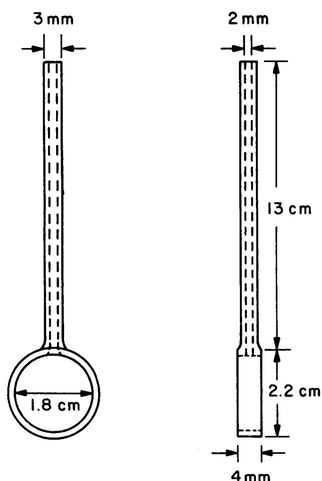

FIG. 1.—Dialysis chamber. A 3-mm OD Pyrex tube was fused to a 2.2-cm OD Pyrex tube. The large tube was then cut to form a thin cylinder with an arm for filling and handling. Using plastic dissolved in amyl acetate as a glue, a VM Millipore filter (50 mμ average pore size, Millipore Filter Corp., Bedford,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

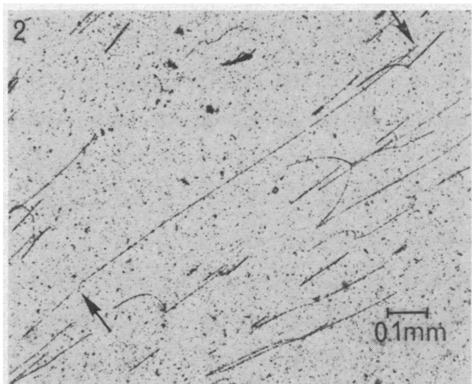

FIG. 2.—Typical DNA autoradiograms. Arrows indicate long autoradiogram. Exposure time was 3 months.

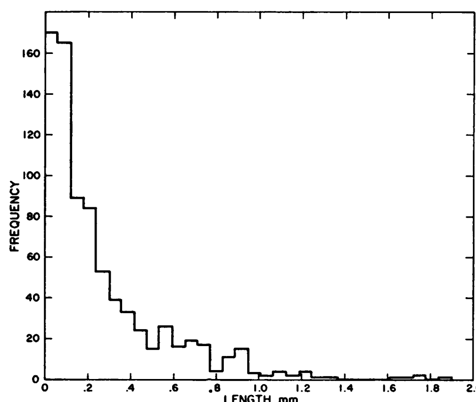

FIG. 3.—Frequency of DNA autoradiograms as a function of length.

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.

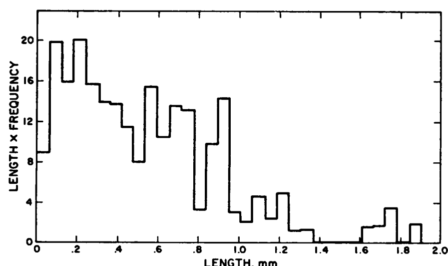

FIG. 4.—Frequency times length of DNA autoradiograms as a function of length. Data are the same as in Fig. 3.

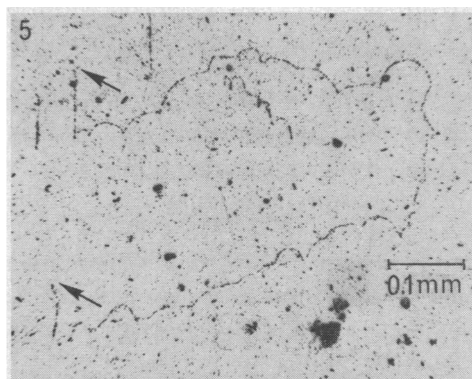

FIG. 5.—A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was 3 months.

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

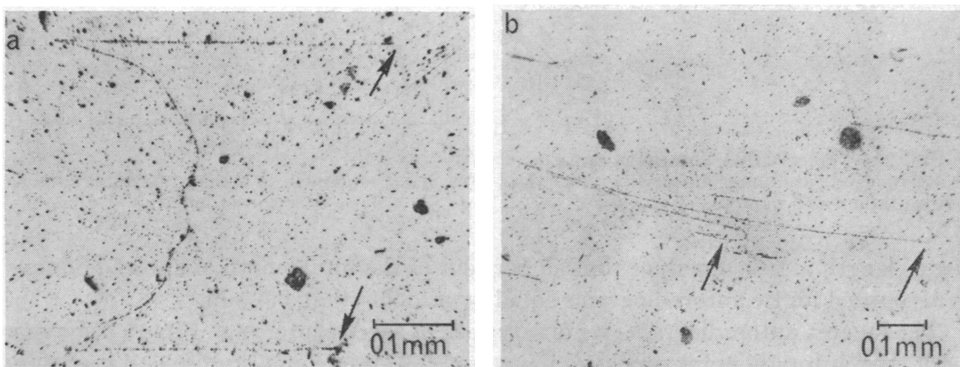

FIG. 6.—(*a*) A DNA autoradiogram 1.4 mm long (between arrows). Exposure time was 3 months. (*b*) A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was $3^1/_2$ months.

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 20×10^{-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 $3.2\text{–}3.6 \times 10^9$ 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.

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A POSSIBLE MECHANISM FOR INITIATION OF PROTEIN SYNTHESIS*

BY TOKUMASA NAKAMOTO AND DANIEL KOLAKOFSKY

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO

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The process by which the information transcribed from DNA to RNA is translated into the amino acid sequences of proteins must include a precise mechanism for initiating synthesis. Specifically, a particular codon of the messenger RNA must serve as the starting point for synthesis of the polypeptide chain. Current descriptions of protein synthesis, however, do not include an established mechanism for chain initiation. This paper proposes such a mechanism, which is in accord with the well-known behavior of protein-synthesizing systems using natural and synthetic messenger RNA. Additional experimental evidence has been obtained in support of this proposal.

The initiation of polypeptide synthesis requires the fulfillment of two conditions,