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Units of DNA Replication in Mammalian Chromosomes[1]

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INTRODUCTION

Studies of DNA replication in chromosomes of mammalian cells clearly indicate that separate sectors of chromosomes replicate simultaneously. This was first shown by autoradiographic studies of metaphase chromosomes (12) and later found to be consistent with the evidence at the molecular level (2, 11, 14, 15). All three of the studies at the molecular level, one by autoradiography (2) and the two others by CsCl density gradient centrifugation gave an upper limit to the size of the unit of replication, which will be referred to as a replicon (13) following the terminology which Jacob and Brenner (8) used for bacteria. The long arm of the X chromosome of Chinese hamster replicates in about 200 minutes, and the chains of DNA grow at the rate of 1–2 microns per minute. Considering the amount of DNA in this arm, there must be at least 130–250 continuously working growing points to complete the DNA in the given time (14, 15). Assuming this is typical of the other chromosomes there must be 5,000–10,000 growing points and at least that number of replicons per cell. Each replicon would have 200–400 microns of DNA if it grew continuously at the indicated rate for 200 minutes. However, Huberman and Riggs (7) have very recently obtained autoradiograms of extended DNA chains which were labeled for 30 minutes with thymidine-^{3}H in cells which had been partially synchronized in DNA synthesis by 12 hours in a medium with fluorodeoxyuridine. These autoradiograms indicate that growing regions are about 30 microns apart along the extended DNA duplex. Other autoradiograms in which the cells continued to grow with thymidine-^{3}H of a decreasing specific activity for an additional 45 minutes produced labeled segments with a lower specific activity at each end. They interpret this to indicate that DNA replication begins at points about 30 microns apart and proceeds in both directions until the growing chains meet, presumably at specific loci. At the rates indicated above and at the rates estimated from the autoradiograms, it would appear that the large units of replication continue growth for less than 200 minutes, which is about one-half the S phase. Furthermore, there is reason to believe that units of replication at least one order of magnitude smaller may exist within each larger unit. The evidence for this statement and some indication of the pattern of control in replication is presented below.

MATERIALS AND METHODS

A strain of Chinese hamster cells (B14FAF 28-G3) was grown in milk dilution bottles on Ham's F-10 medium (5) with 5 percent calf serum and 5 percent fetal calf serum. The medium was also supplemented by adding 4 grams of lactalbumin hydrolysate and 100 mg of glutamine per liter, and twice the usual vitamin content. A density hybrid DNA was produced by inhibiting thymidylate synthesis with fluorodeoxyuridine at 10^{-6} M concentration and supplying bromodeoxyuridine at a concentration of 10^{-5} M. Under these conditions it has been shown that essentially complete substitution of bromouracil for thymine occurs in the newly replicated chains of DNA. All of the DNA will replicate to form hybrid molecules, and these will begin a second round of replication before a significant inhibitory effect on growth is noticed (15).

The DNA was prepared and centrifuged as previously described (6, 15).

RESULTS

When cells are transferred from a crowded culture, allowed 1–2 hours to become attached to the glass in the new bottles, and then blocked with fluorodeoxyuridine for 4–6 hours before releasing with bromodeoxyuridine-^{14}C, the production of hybrid DNA occurs as shown in Chart 1. In such a culture, because of the partial synchrony induced by the crowding before transfer, most cells which synthesize DNA received bromodeoxyuridine from the beginning of the S phase. The DNA was sheared to a molecular weight of 10–20 $\times$ 10^6 daltons before centrifugation in order to produce a symmetric profile of radioactivity. Larger DNA particles frequently trail toward the light side of the gradient when the tube is emptied by piercing the bottom and removing the fractions dropwise. It may be noted that the distribution of radioactivity is nearly symmetric around the peak density which is typical for hybrid particles fully substituted in one chain. The absence of any minor peaks indicates that satellite DNA's are either absent or occur in amounts too small to detect. However, under nearly all other conditions in which substitution of the density label begins during the S phase rather than at its beginning, a peak or shoulder of partially substituted DNA occurs to the left of the unsubstituted band. It usually occurs to the right of center in the profile between hybrid and unsubstituted DNA (9). Recently, we have presented evidence that this intermediate density peak is produced either by breakage, which may sometimes be nonrandom across junctions of substituted and unsubstituted segments, or by the existence of two nearly discrete

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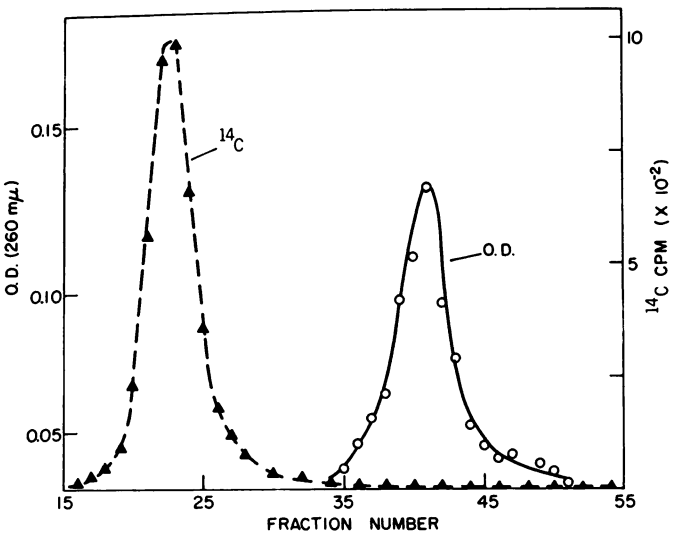

Charts 1–5. Density profiles of DNA in CsCl gradients produced by centrifugation in a Spinco 40.2 rotor at 40,000 rpm for 48 hours; 60 fractions from 3 ml of solution; —▲—▲—, radioactivity; —○—○—, optical density.

Chart 1. DNA extracted from cells grown in bromodeoxyuridine-^{14}C (10^{-5} M) and fluorodeoxyuridine (10^{-6} M) for 3 hours at 37°C, cells substituted bromodeoxyuridine for thymidine from the beginning of the S phase until lysed.

sizes of substituted segments in the DNA (15). The following experiments show that the proportion of such partially substituted DNA fragments can be greatly enhanced by treatments of the cells which reduce the rate of DNA synthesis during the period of substitution of the density label. The occurrence of these partially substituted fragments may give an indication of the size of a unit of replication in chromosomes.

Chart 2 shows the distribution of DNA segments in a CsCl gradient after log phase cells have been growing with bromodeoxyuridine-^{3}H for 20 minutes at 37°C. Cells were placed in Ham's medium with thymidine (10^{-6} M) at the time of subculturing. Additional thymidine (10^{-5} M) and fluorodeoxyuridine (10^{-6} M) were added one hour before substitution to force use of the exogenous thymidine. Ten minutes before substitution, cells were removed from the thymidine-containing medium to deplete the intracellular thymidylate before adding bromodeoxyuridine-^{3}H. Most of the labeled DNA fragments form a nearly Gaussian distribution with a peak at the density position of hybrid DNA. If breakage across junctions were random, the partially substituted DNA should be uniformly distributed between hybrid and unsubstituted DNA. Since partially substituted particles are detected by their radioactivity, specific activity should vary from zero at the right side to a specific activity approaching that of fully substituted hybrid at the left. The distribution of radioactivity from partially substituted DNA should be triangular. The deviation from this expected distribution could be due to nonrandom breakage across all or some junctions, or the persistence of bromouracil segments short enough to be included in a fragment with unsubstituted DNA on each end. Since the rate of chain growth

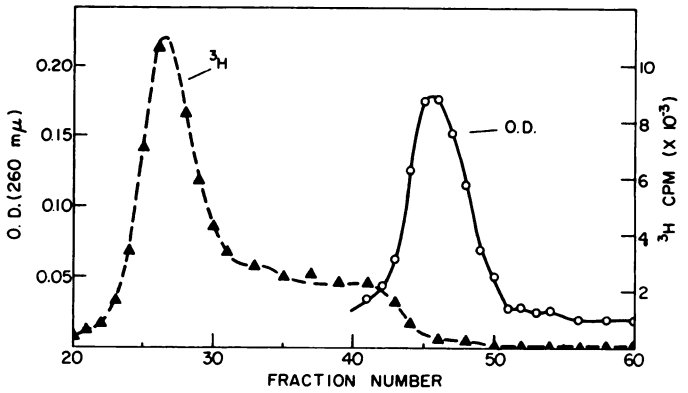

Chart 2. DNA extracted from log phase cells 20 minutes after transfer to a medium with bromodeoxyuridine-^{3}H (10^{-5} M) and fluorodeoxyuridine (10^{-6} M) at 37°C. Cells were depleted of thymidylate by 10 minutes on medium without thymidine after one hour of previous growth on medium with thymidine (10^{-5}M) and fluorodeoxyuridine (10^{-6} M).

has been shown to be 20–40 microns in 20 minutes under these conditions and the fragments are 5–10 microns, such short segments should be rare unless the growth of some chains stopped during the interval. However, in this instance, it is difficult to decide between the alternatives of nonrandom breakage and interrupted growth.

In another experiment, cells handled as described above were lysed at 5, 10, 15, and 20 minutes after beginning growth in bromodeoxyuridine medium. The difference was that the temperature was lowered from 37°C to 31°C within one or two minutes after transfer to bromodeoxyuridine-^{3}H. At five minutes the profile (Chart 3) is nearly typical of cultures grown at 37°C, except that more of the fragments form a peak at a position indicating about 13 percent substitution. Within 10 minutes (Chart 4), fully substituted hybrid particles

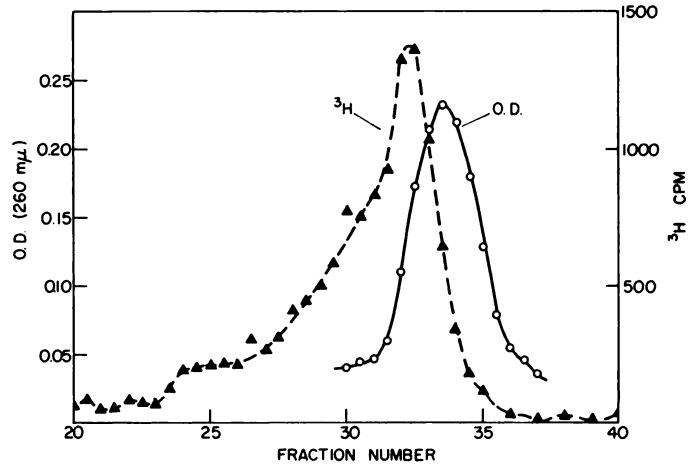

Chart 3. DNA extracted from log phase cells 5 minutes after transfer to medium with bromodeoxyuridine-^{3}H; conditions of growth the same as indicated for Chart 2, except the temperature was lowered to 31°C after transfer to bromodeoxyuridine.

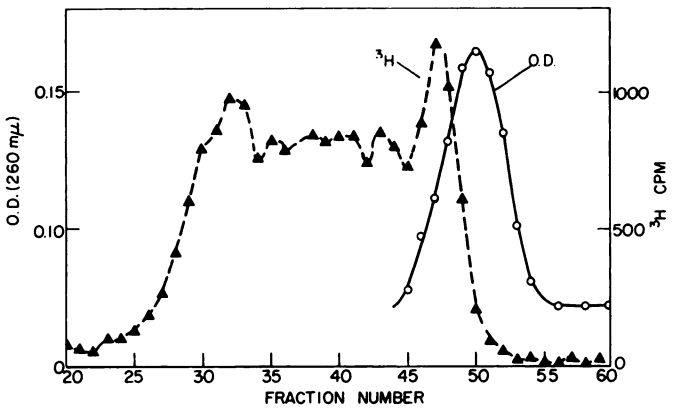

Chart 4. DNA extracted from log phase cells 10 minutes after transfer to medium with bromodeoxyuridine-^{3}H; conditions of growth the same as indicated for Chart 2, except the temperature was lowered to 31°C after transfer to bromodeoxyuridine.

appear but the partially substituted peak persists. By 20 minutes the distribution is strikingly bimodal (Chart 5). Fraction 31 from this profile was dialyzed against 0.015 M NaCl and 0.0015 M Na citrate to remove CsCl, concentrated to 0.1–0.2 ml, and sedimented through a sucrose gradient according to the method of Burgi and Hershey (1). From the distance moved, the average length of the particles was estimated to be about 8 microns (16.6 × 10^6 daltons). Another fraction of DNA from the lighter modal peak of a similar profile was estimated to be composed of particles which averaged about 10 microns in length.

From the profiles shown in Charts 4 and 5, one can draw the conclusion that (*a*) there are substituted segments with two distinct size ranges; (*b*) there is residual thymidylate which

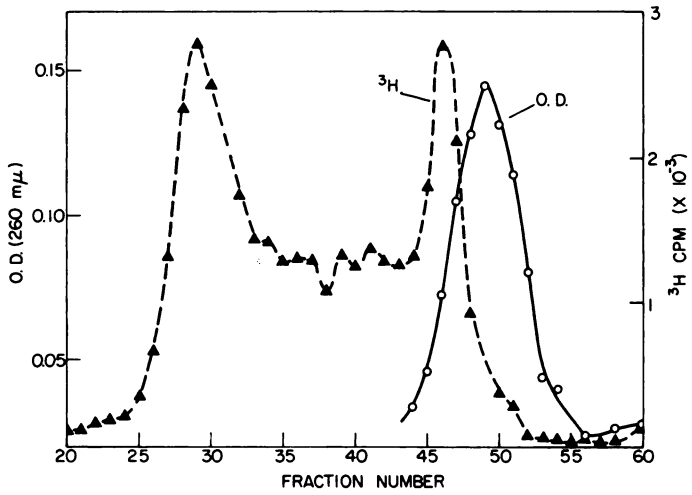

Chart 5. DNA extracted from log phase cells 20 minutes after transfer to medium with bromodeoxyuridine-^{3}H; conditions of growth the same as indicated for Chart 2, except the temperature was lowered to 31°C after transfer to bromodeoxyuridine.

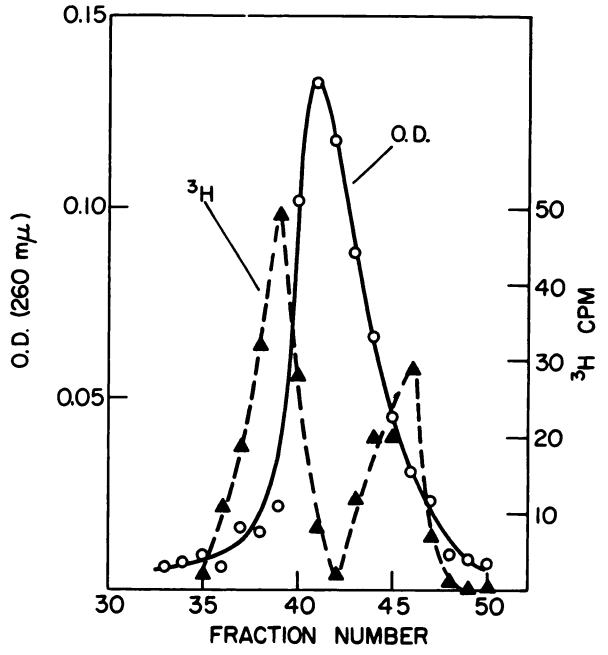

Chart 6. Density profile of a fraction of DNA from the lighter modal peak of a profile similar to Chart 5 rebanded without additional breakage. A peak similar to the one at the right has often, but not consistently, appeared in other rebanded light fractions similar to this one. Its significance is uncertain. —▲—▲—, ^{3}H; —○—○—, optical density.

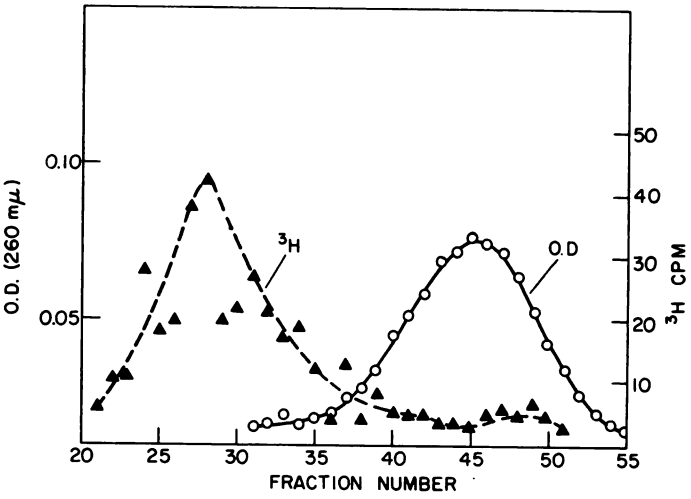

Chart 7. Density profile of a fraction identical to that shown in Chart 6 except that the DNA was broken by sonication with a "Bronwill Biosonik" for 2 minutes with the 0.5-inch probe at an intensity setting of 50 on a scale of 100. The particle size is in the range of 1–2 × 10^6 daltons. —▲—▲—, ^{3}H; —○—○—, optical density.

is quickly depleted before bromodeoxyuridine is utilized; or (*c*) there is some light component attached to the DNA in the lighter modal peak. Rebanding of fractions taken from the lighter peak, with and without additional breakage, indicates that the difference in density is due to two distinct sizes of fully substituted hybrid segments. Another profile of the DNA shown in Chart 5 was prepared several weeks later. The sample was sheared as usual by passage of the solution through a 27-gauge needle. The peak to the right was very much reduced, as would be expected if the decay of ^3H had produced some single chain breaks which reduced the average size of the labeled particles in the CsCl gradient. However, three fractions in the region of the light modal peak were pooled and prepared for rebanding. One-half was rebanded without additional shear except for the small amount involved in preparation of the tube for centrifugation (Chart 6). The other half (Chart 7) was sheared by sonication, producing particles with a molecular weight in the range 1–2 $\times$ 10^6 daltons. It may be seen (Chart 7) that, after this amoun of breakage, most of the labeled DNA bands at a position which approaches the density of fully substituted bromodeoxyuridine hybrid.

The change in rate of synthesis which occurred when the temperature was lowered may have involved some decrease in rate of chain growth, but one striking effect was to block growth of a large fraction of the chains for a period up to 20 minutes. Using a length of 8–10 microns for these partially substituted fragments in the CsCl gradient (estimated as described above by sedimentation in a sucrose gradient), the small bromouracil-substituted segments appear to be about 15 percent substituted in one chain. From these parameters the segments can be estimated to have an average length of 1.2–1.5 microns.

From the narrow range over which the partially substituted particles (Chart 5) are distributed at the modal peak, the conclusion can be drawn that they form a rather discrete population with respect to the size of the substituted segment. This suggests that chain growth stops not at random but after a short determinate growth, perhaps at the end of a replicating unit. If it is assumed that the transition to bromouracil occurs at random in the units which are replicating at the moment of transfer, the length of the bromouracil segments should be one-half the average length of the replicating unit (2.4–3.0 microns of DNA, equivalent to 5–6 $\times$ 10^6 daltons, for an entire replicating unit). If units of this size were replicated at random throughout the chromosome, the result would be that most, if not all, of the DNA would have a density less than hybrid when sheared to the size indicated here. Therefore, if units of this size do exist, they must usually be initiated sequentially from certain chromosomal sites which regulate larger units of the genome.

DISCUSSION

With the information provided in the experiments described here, and with the patterns of replication discovered by means of autoradiography by Huberman and Riggs (7), a fairly clear picture of the patterns of replication in the mammalian chromosome can now be given (Chart 8). Large units of replication called replicons have an average length of about 30 microns (60 $\times$ 10^6 daltons). These begin replication at the midpoint and proceed bidirectionally to the junctions with the two adjacent replicons. However, within each replicon there appear to be subunits with a size range of 2–3 microns. These could be swivel points for the replicating double helix and might be places at which one chain of the duplex is opened. The autoradiograms of Huberman and Riggs (7) indicate that neither the original duplex nor the replicated region opens up until the replicon is essentially complete; they propose that their observations are consistent with a growing fork at which both chains are growing in the same direction. However, it is quite possible that the short units are replicated by growth of one chain at a time so that growth is always from the 3′ hydroxyl end. The small units could then be coupled by the enzymes similar to those now known to exist which repair single chain breaks (3, 4, 10).

Chart 8. Diagram of the distribution and relative size of units of replication along a chromosome segment according to the data from Huberman and Riggs (7). The *brackets* show the large units of replication (replicons) which in Chinese hamster cells average about 30 microns in length. The *arrows* indicate the direction of chain growth according to the bidirectional model of Huberman and Riggs.

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REFERENCES

1. Burgi, E., and Hershey, A. D. Sedimentation Rate as a Measure of Molecular Weight of DNA. Biophys. J., *3:* 309–321, 1963.
2. Cairns, J. Autoradiography of HeLa Cell DNA. J. Mol. Biol., *15:* 372–373, 1966.
3. Gefter, M. L., Becker, A., and Hurwitz, J. The Enzymatic Repair of DNA. I. Formation of Circular λ DNA. Proc. Natl. Acad. Sci. U. S., *58:* 240–247, 1967.
4. Gellert, M. Formation of Covalent Circles of Lambda DNA by *E. coli* Extracts. Proc. Natl. Acad. Sci. U. S., *57:* 148–155, 1967.
5. Ham, R. G. An Improved Nutrient Solution for Diploid Chinese Hamster and Human Cell Lines. Exptl. Cell Res., *29:* 515–526, 1963.
6. Haut, W. F., and Taylor, J. H. Studies of Bromouracil Deoxyriboside Sustitution in DNA of Bean Roots (*Vicia faba*). J. Mol. Biol., *26:* 389–401, 1967.
7. Huberman, J. A., and Riggs, A. D. On the Mechanism of DNA Replication in Mammalian Chromosomes. J. Mol. Biol., in press, 1967.
8. Jacob, F., and Brenner, S. Genetique Physiologique.—Sur la

regulation de la synthese du DNA chez les bacteries: l'hypothese du replicon. Compt. Rend. Acad. Sci., *256:* 298–300, 1963.

9. Miner, P. Replication of DNA in Cultured Chinese Hamster Cells. Thesis, Columbia University, 1967.
10. Olivera, B. M., and Lehman, I. R. Linkage of Polynucleotides through Phosphodiester Bonds by an Enzyme from *Escherichia coli*. Proc. Natl. Acad. Sci. U. S., *57:* 1426–1433, 1967.
11. Painter, R. B., Jermany, D. A., and Rasmussen, R. E. A Method to Determine the Number of DNA Replicating Units in Cultured Mammalian Cells. J. Mol. Biol., *17:* 47–56, 1966.
12. Taylor, J. H. Asynchronous Duplication of Chromosomes in Cultured Cells of Chinese Hamster. J. Biophys. Biochem. Cytol., *7:* 455–464, 1960.
13. Taylor, J. H. DNA Synthesis in Relation to Chromosome Reproduction and the Reunion of Breaks. J. Cellular Comp. Physiol., *62* Suppl. 1: 73–86, 1963.
14. Taylor, J. H. The Regulation of DNA Replication in Chromosomes of Higher Cells. *In:* S. Seno (ed.), Second International Symposium on Cell Chemistry, pp. 231–239. Oxford: Pergamon Press, 1967.
15. Taylor, J. H. Rates of Chain Growth and Units of Replication in DNA of Mammalian Chromosomes. J. Mol. Biol., *31:* 579–594, 1968.