EARLY EVENTS IN THE REPLICATION AND INTEGRATION OF DNA INTO MAMMALIAN CHROMOSOMES

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Received December 23, 1968

Summary.--Analysis of DNA produced in pulses of H^3-thymidine (30 seconds to 2 minutes) indicates that replication of DNA in chromosomes of higher cells proceeds by the production of short segments of perhaps 1000 nucleotides. These segments apparently separate from the template during lysis of cells. However, a large fraction of the DNA remains on the template after a 2-minute pulse. This DNA is linked into chains most of which have sedimentation coefficients in the range of 15-20 S or greater in alkaline sucrose. After a 1-hour chase the new chains have lengths equivalent to the template DNA most of which sediments with a peak at 70-75 S.

A study of some early events in the process of replication indicates that newly synthesized DNA appears in small pieces only a part of which remains on the template during isolation (1-8). The indication that DNA replication proceeded on both template strands simultaneously in the same direction (9, 10) posed the problem of two enzymes and two mechanisms of polymerization since additions would presumably require addition of monomers to both 5' and 3' ends. However, the finding of nearly all of the DNA in short pieces at early times suggests that growth may occur only by addition at the 3' OH ends as observed in vitro (11, 12). The pieces of DNA from bacteria, which are not attached to the template after isolation in neutral salt solutions, have been reported to have sedimentation coefficients from 7 to 11 S. The data of Taylor and Miner (13) suggest that higher cells might replicate DNA in segments 2-3 microns in length. The purpose of the present communication is to report some properties of the short segments isolated

from Chinese hamster cells at early stages in replication and to follow their integration into longer subunits.

Materials and Methods.--A strain of Chinese hamster cells (B14 FAF 28-G3) was grown in Ham's F10 medium as previously described by Taylor (8). About 4×10^5 cells which had been growing in Leighton tubes for 20-24 hours were rinsed for 30 seconds with 10^{-6} M F-dUrd[+] (Calbiochem Corp.) and then pulse labeled with H^3-dTrd 100 μC/ml (sp act 15.9 mC/mM; New England Nuclear Corp.). Medium used for rinse, label, or chase was conditioned on larger cell cultures of the same age as the experimental ones, and manipulations of cultures were carried out in a 37° constant temperature room. The solutions used for lysing cells, shown in Figs. 1-3 contained 0.15 M $NaH\,CO_3$, 0.015 M EDTA, 0.25 percent SDS, and 10 percent sucrose at pH 8.0. The remainder of the cells were lysed in 0.05 M Tris, 5 mg/ml of Chelex (Bio Rad), 0.2 percent SDS and 3 mg/ml pronase (Calbiochem) at pH 8.0. The enzyme was heated at 80° for 10 minutes and preincubated at 37° for at least one hour before adding it to the lysing solution. The cell lysates were incubated with pronase at 37° for 5 hours. DNA was denatured by dropwise addition of 0.4 M NaOH until the solution reached pH 12. The lysates containing one μg or less DNA per sample were layered on top of isokinetic sucrose gradients prepared as described by Taylor (14) in tubes for the Spinco SW 27 rotor. The sucrose gradients were fractionated by puncturing the nitrocellulose tubes and pumping 40 percent sucrose into the bottom of the tubes. Fractions were precipitated by the addition of 2 drops of 0.01 percent albumin and TCA to 5 percent final concentration and then trapped on nitrocellulose filters (Millipore Filter Corp.). The radio-

[+]Abbreviations used: F-dUrd, fluorodeoxyuridine; dTrd, deoxythymidine; SDS, sodium dodecyl sulfate; TCA, trichloroaceticacid

activity of the samples was determined with a liquid scintillation counter. Hydroxyapatite was prepared according to Miyazawa and Thomas (15). Pulse labeled DNA was diluted in 0.05 M phosphate buffer, pH 6.8, and pipetted on the hydroxyapatite column. The column was washed once with 0.05 M phosphate buffer then the adsorbed DNA was eluted stepwise with 2 ml aliquots of phosphate buffer. The fractions were assayed by precipitation and filtration through millipore filters as described above.

Results and Discussion.--Pulse-labeling of asynchronous, rapidly growing Chinese hamster cell cultures revealed that the DNA was replicated

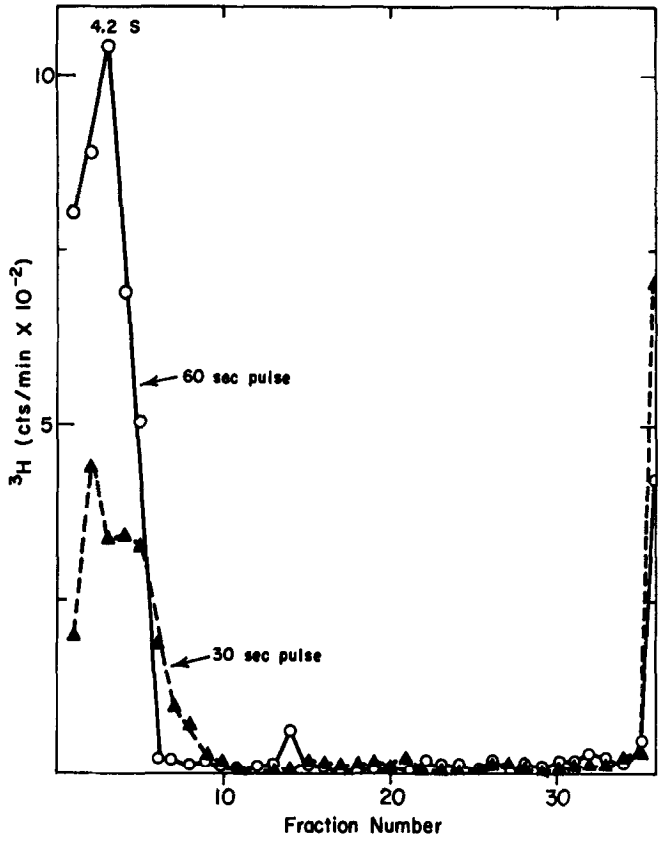

Fig. 1--Sedimentation profiles of two DNA preparations in alkaline sucrose gradients. Cells were pulse labeled for 30 and 60 seconds, respectively, and lysed directly on the glass surface to which they were attached. Centrifuged at 26,500 rpm for 9 hours.

as small, slowly sedimenting pieces in both alkaline and neutral sucrose gradients. When the labeling period, utilizing tritiated thymidine, did not exceed two minutes as reported earlier (14, 16) most of the labeled DNA remained near the top of the gradients and an accumulation of the short pieces could be detected (Fig. 1). The template DNA as previously reported (14) formed a peak at 70-75 S, or was found in the bottom of the tube when centrifugation was continued long enough to move the small pieces well into the gradient.

In a series of short pulse-chase experiments the small pieces were shown to become attached to longer pieces, but not at a rate expected if the growing chains to which small pieces are attached exist in a uniform size distribution from the smallest pieces to the size of the template. As may be seen in Figs. 2 and 3, the new chains increase in size with time, but a large fraction of the growing chains form a prominent peak (up to 50% of the label) as if the pulse partially synchronized chain growth by initiating new chains or somehow produced a gap between the growing chains which existed at the moment of the pulse and those which formed afterward. However, it may be noted that some of the pulse labeled DNA also attached to pieces which form a nearly uniform distribution from the peak of small pieces to the bottom of the gradient. Previous experiments (14, 16) have shown that chain growth from the beginning of the S-phase in synchronized cells is linear for at least one-half hour at one micron per minute. However, elongation of the pulse labeled pieces in these asynchronous cultures appears to be nearly two times as fast as shown in Fig. 2. The 26 S peak in this figure indicates that chains attain an average length of about 15 microns in eight minutes. A one-hour chase produced labeled chains which sedimented along with the template (70-75 S) as well as some pieces of about one-half

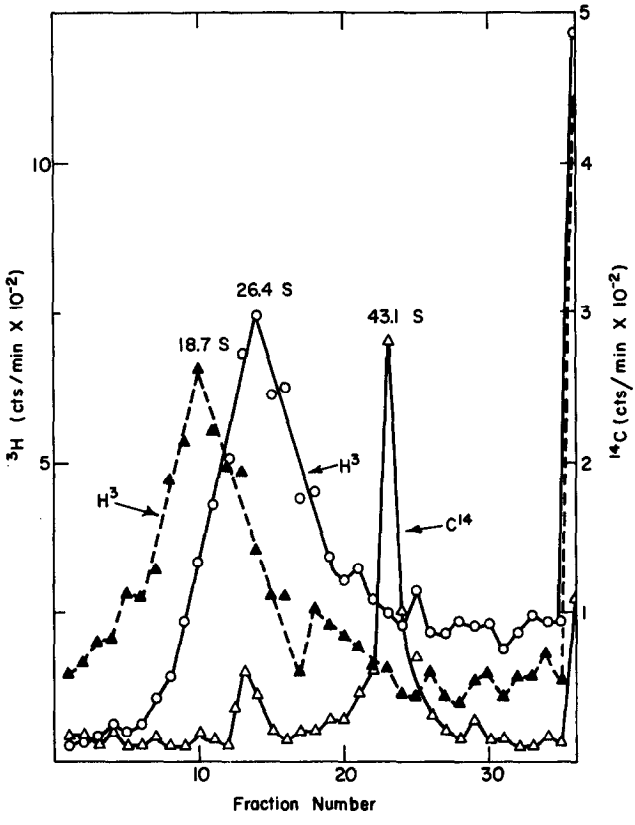

Fig. 2--Sedimentation profiles of two DNA preparations in alkaline sucrose gradients. Cells were pulse labeled for 30 seconds with H^3-thymidine, rinsed with medium containing unlabeled thymidine and grown for 2 and 7 minutes, respectively. C^{14}-labeled phage T4 was lysed along with the cells as a marker. Centrifuged at 26,500 rpm for 12 hours. —▲--▲—, 2 minute chase; —○—○—○—, 7 minute chase; —△—△—△—, T4 phage DNA.

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that size (54 S) which are perhaps new strands which have separated from the template (Fig. 3).

In order to learn more about the small pieces of newly replicated DNA, 2-minute pulse-labeled DNA preparations were adsorbed on hydroxyapatite and eluted stepwise with increasing concentrations of phosphate (15). Fig. 4 depicts the elution pattern of the H^3-labeled, newly synthesized DNA. Three major fractions of DNA were obtained. Fraction I did not adsorb to

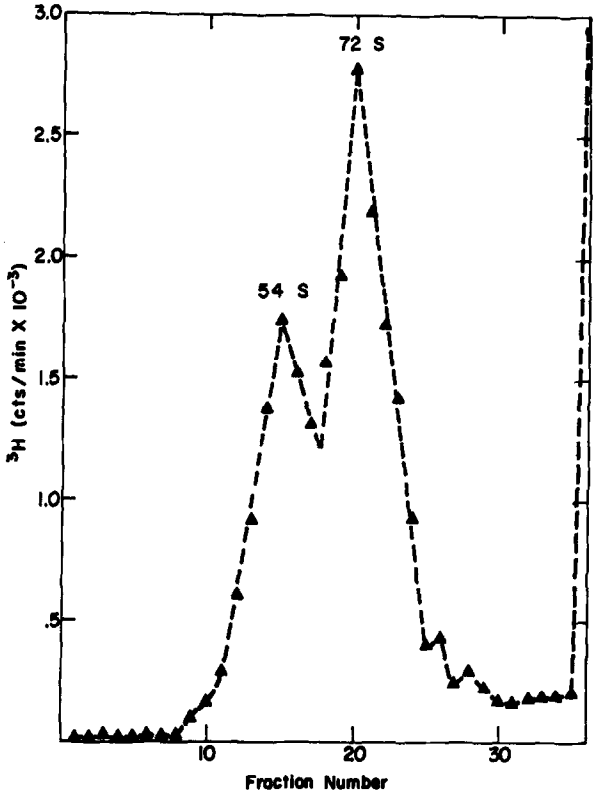

Fig. 3--Sedimentation profile of a DNA preparation in an alkaline sucrose gradient. Cells were pulse labeled for 3 minutes, rinsed and grown in unlabeled thymidine for one hour. Centrifuged at 27,000 rpm for 5 hours.

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the hydroxyapatite and probably consists of very small pieces of single stranded DNA; fraction II came off with a peak at 0.14 M phosphate and should consist of single stranded DNA; and fraction III eluted with a peak at 0.20 M phosphate. It appears to be composed of double stranded pieces of intermediate length. About 9 percent of the radioactivity either remained on the hydroxyapatite (4%) or eluted with 0.5 M phosphate (5%).

Fractions II and III were analyzed by sedimentation in alkaline and neutral sucrose gradients. Fraction II sedimented with a peak at about the

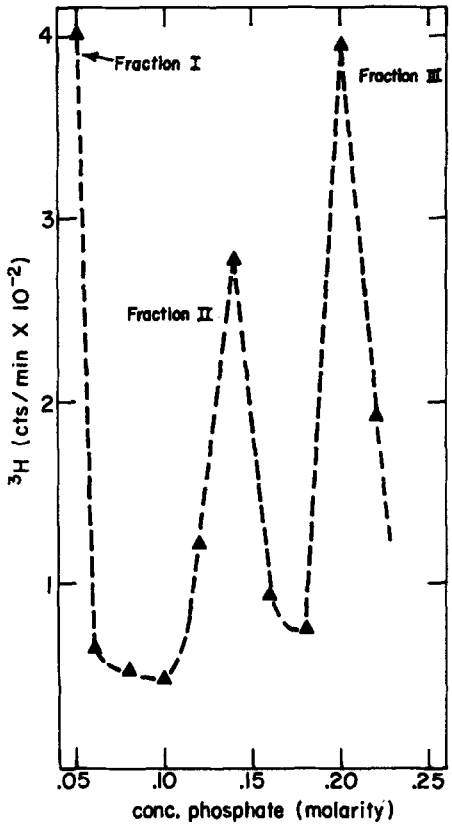

Fig. 4--Chart showing the elution of pulse labeled Chinese hamster DNA from a hydroxyapatite column with increasing concentrations of phosphate buffer. See text for details.

same position as the total labeled DNA after 30- and 60-second pulses. The longer period of sedimentation confirmed the original observation that a single peak usually was formed at the position expected for 4-6 S particles. However, the leading edge of the labeled pieces moved out far enough to indicate that some of the pieces might have a sedimentation coefficient two times those at the peak. The sedimentation profile of fraction II was similar in alkaline and neutral gradients with perhaps enough increase in the neutral gradient to indicate particle interaction even at this very low concentration.

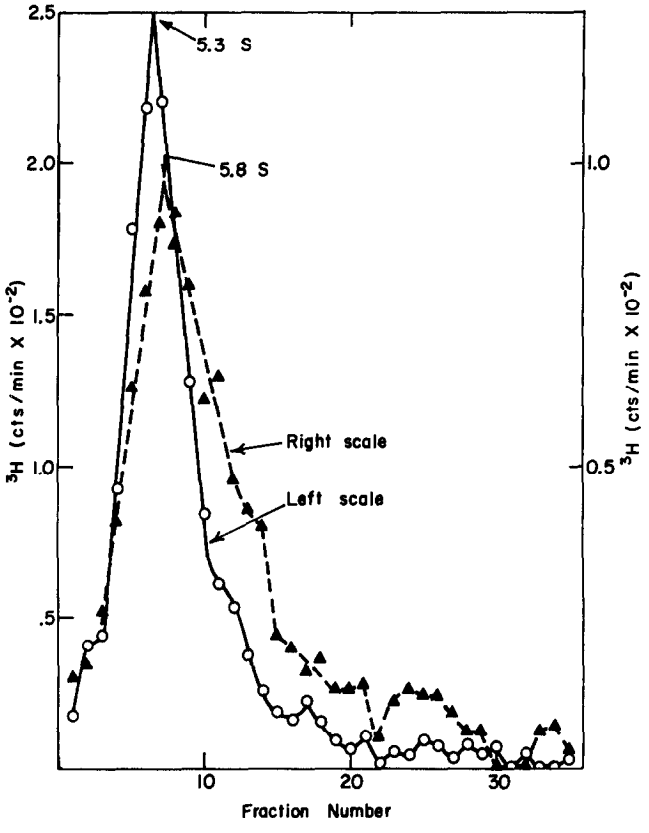

Fig. 5--Sedimentation profile of pulse labeled DNA (Fraction II from hydroxyapatite column) in alkaline sucrose gradient, —O—O—O— ; and in neutral sucrose gradient, —▲—▲— . Centrifuged at 27,000 rpm for 20 hours.

However, fraction III appeared to be not only larger but denaturable (Fig. 6). It is probably composed of newly synthesized DNA which was attached to pre-existing, longer chains which were on the template before the pulse label. The pieces have a wide range of sizes but a peak in the range of 18 S is apparent in the alkaline gradient (compare with the peak in Fig. 2).

The tentative model which emerges from these results and previous reports is that of relatively small pieces of DNA, perhaps with about 1000 nucleotides, which are replicated and accumulated for a minute or two

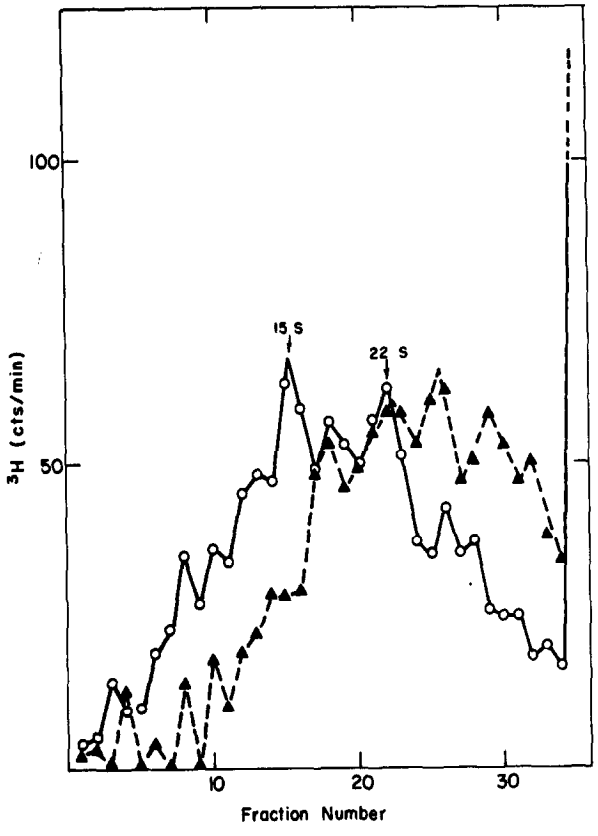

Fig. 6--Sedimentation profile of pulse labeled DNA (Fraction III) in alkaline sucrose gradient, —o—o—o— ; and in neutral sucrose gradient, —▲—▲— . Centrifuged at 27,000 rpm for 20 hours.

before being joined to longer chains. These pieces are easily removed from the template during lysis until they are attached to longer chains. The accumulation of chains which have sedimentation coefficients of 15-20 S within two minutes is also consistent with an intermediate sized subunit which was indicated by previous studies (8, 13) and has recently been isolated from this cell line in stationary phase cells (17). The DNA in the stationary phase cells appears to have non-adjacent "nicks" in alternate strands similar to a model of chromosome structure previously presented (18).

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