THE SYNTHESIS AND ASSEMBLY OF DNA SUBUNITS IN ISOLATED HELA CELL NUCLEI[1]

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Summary. Nuclei from synchronized HeLa cells in S-phase continue to synthesize DNA *in vitro* at or near the *in vivo* replicating sites. Sedimentation of the product in an alkaline sucrose gradient reveals 10 and 24S segments in addition to the 40S non-replicating bulk DNA. Pulse chase experiments indicate that the 10S DNA is a precursor of the 24S product. ATP and the soluble protein fraction from the cytoplasm of S-phase cells support the accumulation of the 24S product.

DNA replication in living mammalian cells (1,2), as in the case of bacteria and phage (3,4,5) appears to be a discontinuous phenomenon involving the initial formation of short segments which are subsequently linked together to yield full size, interphase or non-replicating DNA. DNA replication thus appears to consist of at least two processes: the polymerization of nucleotides and the ligation of contiguous oligonucleotides into a chain. The development of a subcellular system from synchronized HeLa cells which continues the nuclear synthesis of DNA (6) has opened the way to study these processes *in vitro* as well as the control mechanisms which regulate DNA synthesis. The present experiments describe the step-wise synthesis of DNA *in vitro* and the participation of ATP and a soluble protein fraction (CF) in the system.

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Materials and Methods. HeLa cells were maintained and synchronized with amethopterin and adenosine in shaker cultures as described (7). Two or 3 hours after initiating DNA synthesis with thymidine (3 µg per 10^6 cells) or bromodeoxyuridine (10^{-5} M) the cells were harvested and nuclei prepared by hypotonic lysis of the cells as described earlier (6) except that all solutions contained 1 mM EDTA and 2 mM 2-mercaptoethanol. After the nuclei were pelleted from the broken cell suspension (800 x g for 5 minutes) the supernatant was used to prepare the soluble protein fraction (CF) by ultracentrifugation (100,000 x g for 1 hour at 0°C). CF was stored at -80°C until used in the nuclear assays. The nuclei were washed in 60 mM Tris-HCl, pH 8, containing 5 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM EDTA, 11 mM glucose, and 60 mM NaCl. Depending on the experiment, the washed nuclei were resuspended in CF or an equivalent amount of salt solution (30 x 10^6 nuclei per 1.5 ml) prior to assay. The final concentrations of the components in the basic assay system were 9 mM $MgCl_2$, 1mM EDTA, 2mM 2-mercaptoethanol, 5.3 mM potassium phosphate, 24 mM glucose, 92.5 mM NaCl, 92.5 mM Tris-HCl buffer, pH 8, and 0.5 mM each of dATP, dGTP, dCTP, and TTP. When present, ATP was at a concentration of 5 mM and H^3-TTP (New England Nuclear, 11 C per mMole) at a level of 20-70 µC/ml.

Incubations were performed at 37°C for various times and the reaction was stopped by placing the tubes in an ice bath. Nuclei were then pelleted from the reaction mixture and extracted successively with 2 ml aliquots of cold ethanol, $CHCl_3$-isoamyl alcohol (2:1) and ether (2 x). The residue was suspended in 2.0 ml of lysis buffer (8) containing pronase (Calbiochem., 1 mg per ml, autodigested for 1/2 hour at 37°C) and the mixture digested for 2-3 hours at 37°C. After dialysis for 12 hours against 15 mM NaCl-1.5 mM sodium citrate, the samples were deproteinized with $CHCl_3$-Isoamyl alcohol (8), and the DNA precipitated with an equal volume of cold ethanol. The product was dissolved immediately in 15 mM NaCl-1.5 mM sodium citrate for CsCl gradient centrifugation or 0.1 M NaOH for alkaline sucrose gradient

centrifugation.

For the alkaline sucrose gradients, 0.5 to 1.0 ml of DNA solution (25 - 150 µg) was placed on a linear gradient (5-20% sucrose in 0.1 M NaOH, 0.9 M NaCl, 0.001 M EDTA). For the determination of sedimentation coefficients, isokinetic gradients (5-22% sucrose in 0.1 M NaOH, 0.9 M NaCl, 0.001 M EDTA) were utilized. Samples were centrifuged in the SW 25.1 rotor at 22 K rpm for 12-16 hours at 0°C. Gradient fractions were precipitated with cold 4% perchloric acid in the presence of 250 µg carrier DNA. Precipitates were hydrolyzed in 0.5 M perchloric acid at 90°C and 0.5 ml aliquots were counted (6).

Results and Discussion. As previously reported (6) DNA synthesis in isolated HeLa nuclei reflects the in vivo competency of the nuclei for DNA synthesis and appears to be a continuation of this process in vitro. All 4 deoxyribonucleotide triphosphates are required and the product which is DNase sensitive has physical properties in common with replicative DNA from living cells[9]. To demonstrate that the in vitro synthesis of DNA continues at the sites of in vivo DNA replication, the following type of experiment was carried out. Synchronized cultures were reversed with bromodeoxyuridine and allowed to synthesize DNA for 3 hours with this analogue of thymidine as a density marker. At this point the cells were harvested and isolated nuclei incubated in the in vitro system in the presence of ATP, CF and H^3-TTP. The DNA was then extracted and centrifuged in a CsCl gradient. The majority of the radioactivity from the H^3-TTP incorporated in vitro was confined to the heavy, Budr substituted DNA; only a small fraction of the counts centrifuged with the normal density DNA (Figure 1). When the nuclei were prepared from thymidine-reversed cells all of the radioactivity incorporated in vitro centrifuged with the normal density DNA (Figure 2). These data strongly support the conclusion that DNA synthesis in vitro is largely a continuation of synthesis at the replicative sites which were active in vivo; however, a small amount of initiation at new sites may have been achieved as well.

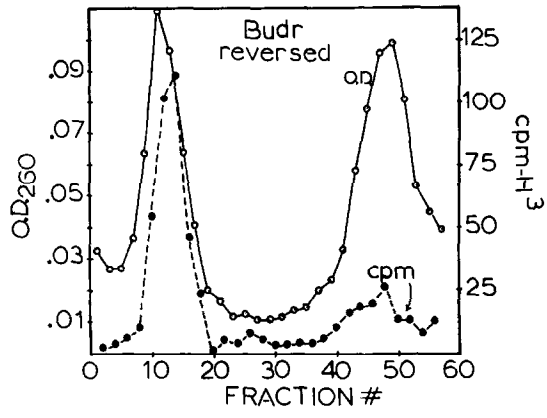

Figure 1. H^3-TTP incorporation into DNA in nuclei from cells reversed with Budr. Three hundred µg DNA or less in 3.4 ml of buffer was mixed with 4.375 g CsCl. Samples were centrifuged in the No. 50 fixed angle rotor according to Flamm, Bond and Burr (10). Five drop fractions were collected and alternate fractions assayed for $O.D._{260}$ or H^3 by a filter disc method (6).

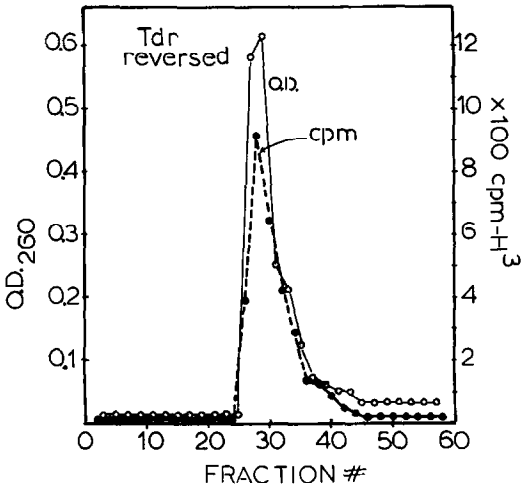

Figure 2. H^3-TTP incorporation into DNA in nuclei from thymidine reversed cells. The protocol was the same as in Fig. 1.

To study the progression of the <u>in vitro</u> synthesis the isolated DNA was analyzed by sedimentation through an alkaline sucrose gradient. For this purpose nuclei were first pre-incubated in the basic system with ATP and CF in the absence of any radioactivity; they were then subjected to a 5 minute pulse with 100 µC H^3-TTP. One half of the reaction mixtures were terminated

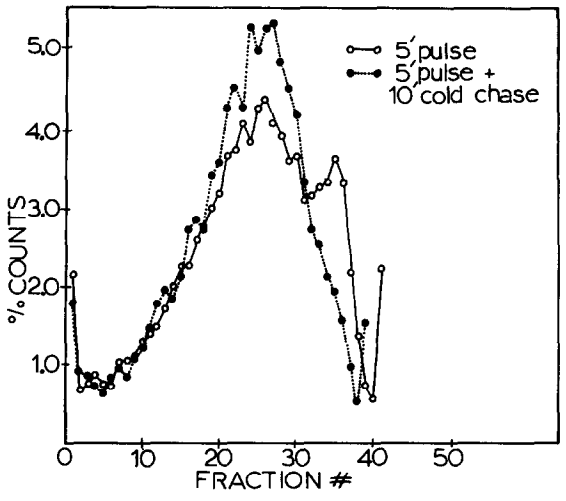

Figure 3. Alkaline sucrose gradient fractionation of DNA synthesized *in vitro* during a five minute pulse with H^3-TTP and following a 10 minute chase with non-labeled TTP. Samples were centrifuged for 16 hours. Forty-drop fractions were collected by dripping through a puncture in the bottom of the tube.

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while the other half received a 20-fold excess of non-isotopic TTP and were allowed to synthesize DNA for another 10 minutes. Centrifugation of the isolated DNA products through alkaline sucrose gradients revealed two size modes of DNA labeled during the 5 minute pulse, corresponding to 10S and 24S in size (Figure 3); during the cold chase the 10S DNA disappeared and a corresponding amount of radioactivity appeared in the 24S peak. Since the specific activity of the isolated DNA increased from 252,000 dpm/mg to 301,000 dpm/mg, it is unlikely that the 10S DNA was degraded. These data support the conclusion that the DNA synthesis in isolated nuclei, as well as in intact cells, proceeds through the synthesis of short segments (10S) which are linked together to make larger units (24S).

To examine the role of CF and ATP in this process DNA synthesized in the presence and absence of these factors was similarly analyzed in alkaline sucrose gradients. In addition to decreasing the total incorporation of H^3-TTP by 86%, the omission of both CF and ATP resulted in a predominance of the 10S size DNA (Figure 4a). Adding CF or ATP separately caused a shift towards

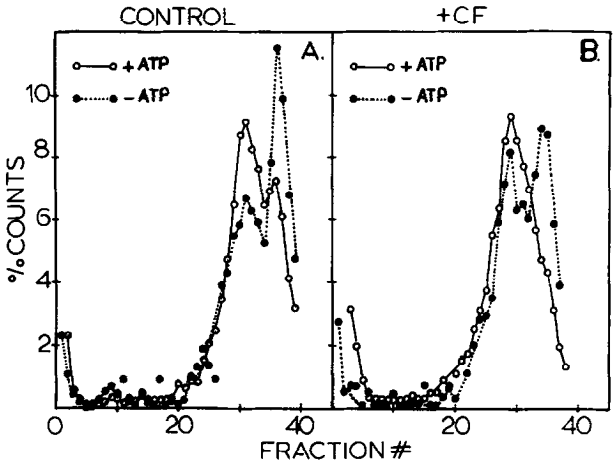

Figure 4. The effect of ATP and or CF on the size of the DNA synthesized in vitro. Nuclei were incubated for 25 minutes in the basic assay system with additions as indicated. A. The effect of ATP in the absence of CF. B. The effect of CF in the presence and absence of ATP.

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the 24S size (Figure 4b) although only a partial restration of the full synthetic potential of the nuclei was achieved (17% and 47% respectively). When CF was omitted in a pulse chase experiment as described in Figure 3, conversion of the 10S to the 24S DNA failed to occur.

Brief exposures of HeLa cells to radioactive thymidine results in the preferential labeling of short DNA segments (10S) which are subsequently converted to larger units (1). To test further whether or not the nuclear system continues the natural mechanism of DNA synthesis, the ability of isolated nuclei to convert 10S DNA which was labeled in vivo to 24S DNA was tested. As shown in Figure 5 incubation of the nuclei in the in vitro system resulted in a conversion of the in vivo labeled 10S DNA to the 24S DNA size. This conversion occurred whether or not CF or ATP were present in the system. Utilizing the C^{14} labelled DNA as a control on recovery, it was observed that the ratio of H^3 to C^{14} in the DNA rose slightly during the in vitro incubation (i.e. 1.00 to 1.07) suggesting that more of the pulse labelled DNA was re-coverable after the incubation.

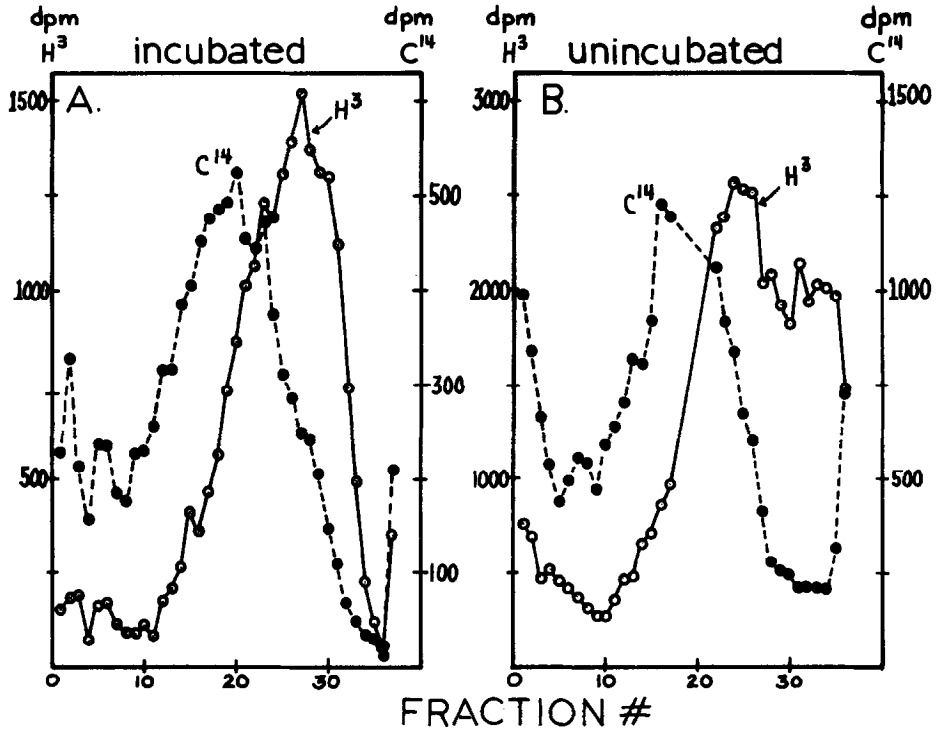

Figure 5. The transformation of 10S DNA synthesized in vivo into the 24S species in isolated nuclei. Randomly growing cells were first labeled uniformly with 2-C^{14} thymidine (0.20 µc, 1 mC/mMole) and then synchronized for experimentation. Three hours after initiation of DNA synthesis by reversal of the thymidineless state, a 30 second pulse with H^3-thymidine (250 µC, 11C/mMole, Schwarz) was given followed by hydroxyurea (2 X 10^{-3}M) and immediate cooling of the cultures. The nuclei were isolated and incubated for 5 min in the DNA synthesis system in the presence and absence of CF and ATP. The DNA was extracted and analyzed by sedimentation in alkaline sucrose gradients. A. DNA extracted from incubated nuclei. B. DNA from nuclei which were not incubated.

These results support the conclusion that the stepwie synthesis of DNA, which is characteristic of the living mammalian cell, has been reconstituted in isolated nuclei. This process involves the synthesis of short segments of DNA which are subsequently linked into larger units. Both CF and ATP are required for the optimal operation of the cell free system; it appears likely that these factors support the initiation of synthesis in contiguous segments and the ligation of these units, respectively.

References

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