DNA Synthesis in Isolated HeLa Cell Nuclei. Evidence for in Vitro Initiation of Synthesis of Small Pieces of DNA and Their Subsequent Ligation†

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ABSTRACT: Optimum conditions for a DNA synthesizing system based on isolated nuclei have been described (Krokan, H., Bjørklid, E., and Prydz, H. (1975), *Biochemistry*, preceding paper in this issue) [^{3}H]TTP-labeled nascent DNA produced during very short pulses was analyzed by centrifugation in alkaline sucrose gradients. More than 80% of the radioactivity appeared in 2-4S pieces (primary DNA pieces). It would therefore seem that the synthesis of DNA is discontinuous both in the 5′→3′ and in the 3′→5′ directions. The size of the primary DNA pieces increases from 2-4 S up to 14 S with increasing pulse length. Evidence is presented that this increase is not caused by ligation between 2-4S primary pieces. Pulse-chase experiments showed that in this nuclear system primary pieces were ligated to a product generally larger than 30 S. Evidence is also given for the initiation of primary DNA pieces in vitro.

In mammalian cells DNA synthesis takes place within separate, tandemly joined replication units of average size 30 μ (corresponding to 6×10^7 daltons or about 70 S) (Huberman and Riggs, 1968). Within each replication unit the synthetic process seems to be discontinuous in a way analogous to that in procaryotic cells (Okazaki et al., 1968), where DNA is synthesized in pieces of about 9 S (Okazaki fragments). In this paper the small fragments which are synthesized after short pulses will be called "primary DNA pieces" and the "intermediate strands" (Brewer et al., 1974) will be called "secondary DNA pieces".

Different sizes for the primary DNA pieces have been reported in various eucaryotic cells, and indeed some workers have been unable to detect such pieces at all (Brewer et al., 1974; Hyodo et al., 1970). The failure to find primary DNA pieces in *Physarum polycephalum* (Brewer et al., 1974) probably arises from the long pulse times used (>4 min), since DNA pieces of around 5-6 S have been detected after pulses of 30-60 sec (S. Funderud and F. Haugli, personal communication). In HeLa and CHO cells (Huberman and Horwitz, 1973) and in P-815 mouse fibroblasts (Gautschi and Clarkson, 1975) primary DNA pieces of about 100 nucleotides (about 3 S) have been found. In Ehrlich ascites cells larger pieces (about 1000 nucleotides or 9 S) have been described (Goldstein and Rutman, 1973). In these three papers all the radioactivity present in DNA after a short pulse was found in primary DNA pieces, indicating that the DNA synthesis was discontinuous in both the 5′→3′ and the 3′→5′ direction. Recently, discontinuous DNA synthesis has been reported to take place in nuclei isolated from HeLa and CHO cells. Friedman (1974) found primary DNA pieces of 5-6 S in the absence of ATP in the incubation mixture and 8-10 S pieces in its presence. Hershey and Taylor (1974) reported fragments of 4-6 S. The results reported in the two latter papers suggest a model involving discontinuous synthesis on one strand and continuous synthesis on the other.

We report evidence for discontinuous DNA synthesis on both strands and for in vitro initiation and ligation of primary DNA pieces in a HeLa cell nuclear system which has been shown to have several in vivo like properties (Krokan et al., 1975).

Materials and Methods

Cell Culture and Isolation of Nuclei. The cells were cultivated, maintained, and synchronized, and the nuclei isolated 3 hr after entry into S phase as described (Krokan et al., 1975). Freshly isolated nuclei were incubated as described in the legends to the figures. The test mixture contained 65 m*M* NH_4Cl, 65 m*M* Tris-HCl (pH 8.1) (20°), 10% glycerol, 50 m*M* glucose, 0.05 m*M* CTP, UTP, and GTP, 0.1 m*M* dATP, dGTP, and dCTP, 5 μM [^{3}H]TTP (final specific activity, 0.05-30 mCi/μmol), 10.7 m*M* $MgCl_2$, and 10 m*M* ATP if not otherwise stated.

Preparation of P2 DNA. The coliphage P2 was grown as described by Lindqvist (1971) and P2 DNA prepared by phenol extraction. The P2 DNA was used as a size marker in alkaline sucrose gradients. The molecular weight of alkaline single-stranded P2 DNA is 1.1×10^7. The calculated sedimentation coefficient was 34 S (Studier, 1965).

Lysis of Cell Nuclei. The nuclei were lysed in ice-cold alkaline buffer containing 0.4 *N* NaOH, 1% *N*-lauroylsarcosine, and 0.01 *M* EDTA. Lysis was immediate, but the lysate was left at 4° for at least 2 hr before centrifugation.

Alkaline Sucrose Gradients. Alkaline sucrose gradients (5-20%) were centrifuged in polyallomer tubes in an SW 27 rotor as indicated in the legends to the figures. The gradient contained 5-20% sucrose, 0.1 *N* NaCl, 0.4 *N* NaOH, 0.1% *N*-lauroylsarcosine, and 10 m*M* EDTA. The alkaline nuclear lysates (1 ml) were layered on top of the gradients, avoiding shear. The loaded gradients were stored for 1-2 hr at 4° before the run. ^{14}C-Labeled P2 DNA was used as a marker in all gradients. The gradients were fractionated from the top by a Buchler Auto Densi-flow apparatus connected to an LKB peristaltic pump and an ISCO fraction collector. The shortest possible connecting tubes were used. The bottoms of the centrifuge tubes were washed with 0.1 *N* NaOH to remove adherent material and the radioactivity

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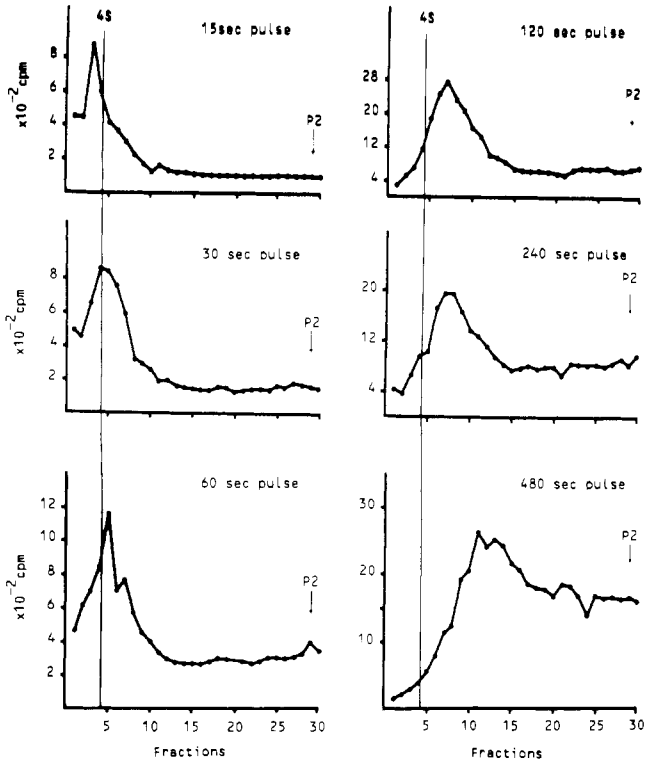

FIGURE 1: Alkaline sucrose gradients of pulse-labeled DNA from isolated nuclei. Nuclei were incubated without ribo- and deoxyribonucleotides for 60 sec at 37° and then labeled by the addition of ribonucleoside and deoxyribonucleoside triphosphates (final concentration of [^{3}H]TTP, 5 μM; specific activity, 7.2 mCi/μmol). The reaction was stopped by addition of ice-cold alkaline lysis buffer. The lysates, each containing about 40 μg of DNA, were layered on top of the alkaline sucrose gradients and centrifuged for 19 hr at 4° and 27,000 rpm. Thirty-five to thirty-six fractions were collected (only 30 fractions shown). The bottom fraction contained 12, 6, 38, 39, 41, and 42% of the total radioactivity in the respective gradients.

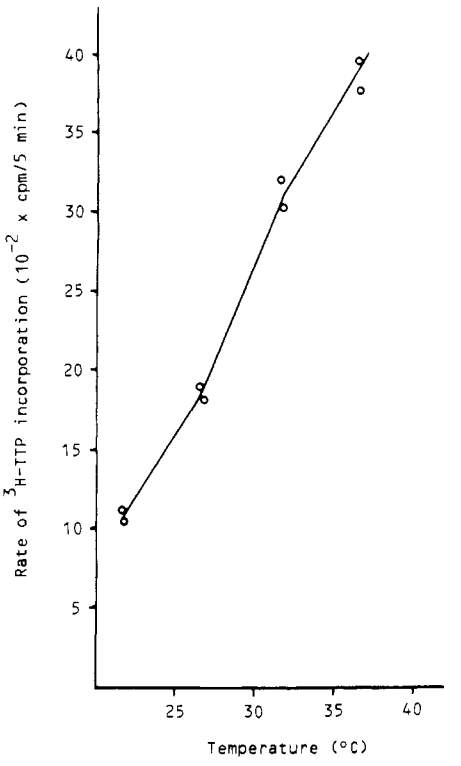

FIGURE 2: Effect of temperature on the rate of DNA synthesis. Nuclei were incubated for 5 min. Final concentration of [^{3}H]TTP in test mixture was 100 μM; specific activity, 50 μCi/μmol.

in the washing fluid was measured. In certain experiments an aliquot was recentrifuged. The walls of the tubes were washed and the washing fluid was counted.

Assay of [^{3}H]TTP Incorporation. The fractions were precipitated by addition of 2 ml of 20% Cl_3CCOOH containing 20 mM sodium pyrophosphate. Albumin (0.4 mg per fraction) was added as a carrier. The fractions were collected on Whatman GF/C filters and washed 6 times with 10 ml of 5% Cl_3CCOOH containing 20 mM sodium pyrophosphate and once with 10 ml of ethanol. The filters were dried, incubated with 200 μl of toluene at 50° for 1 hr, and counted in 10 ml of Instagel in a Packard Tri-Carb liquid scintillation spectrometer. DNA was determined by the method of Burton (1956).

Results

Pulse Labeling of Isolated Nuclei. The first questions asked were: (i) is the DNA synthesis in the present nuclear system discontinuous in the sense that primary DNA pieces are made? (ii) Do these pieces have a discrete size? (iii) How much of the [^{3}H]TTP is incorporated into such pieces after a short pulse?

Nuclei freshly isolated from synchronized cells were preincubated for 60 sec in the test mixture without nucleotides. The nuclei were then pulse labeled for different time intervals by addition of a nucleotide mixture. The incubation was terminated by addition of alkaline lysis buffer and the lysates centrifuged in alkaline sucrose gradients. Low molecular weight single-stranded DNA pieces were synthesized in isolated nuclei (Figure 1). These were taken to be primary DNA pieces. The size of these pieces depended on the pulse length. They increased from 2-4 S (or 1-4 × 10^4 daltons) to about 14 S (about 10^6 daltons) when the pulse length increased from 15 to 480 sec. The larger DNA pieces might be the result of ligation between smaller primary DNA pieces, but for reasons given later this is improbable.

To study the situation after very brief effective pulses, experiments were carried out at lower temperature. From the rate of incorporation at various temperatures (Figure 2) an Arrhenius plot was made and the activation energy was calculated to be 72.8 J/mol. When isolated nuclei were pulse labeled for 30 sec at 22 or 27°, about 80% of the label was found in 2-4S primary DNA pieces (Figure 3). About 10% of the total label incorporated sedimented to the bottom of the centrifuge tube in alkaline sucrose gradients. When recentrifuged under identical conditions 9-10% of this label appeared in the uppermost eight fractions of the gradient. The rest of the material sedimented further down, mainly to the lower third of the gradient. Most of the label in the bottom fraction was therefore probably not due to technical artifacts. To obtain accurate estimates of the background label, nuclei kept at 0° and lysed immediately after mixing with the test mixture were submitted to gradient centrifugation. This was necessary since some acid-precipitable label not present in DNA was regularly detected in such control gradients, probably due to binding of [^{3}H]TTP to proteins in the lysate (Figure 3).

When the pulse time increased, a decreasing part of the total label incorporated into DNA was found in the primary DNA pieces (Table I). More label appeared in DNA larger than about 30 S. This may be explained by a ligation process. Thus, increased pulse time was associated with an increase in the size of the primary DNA pieces and a decrease

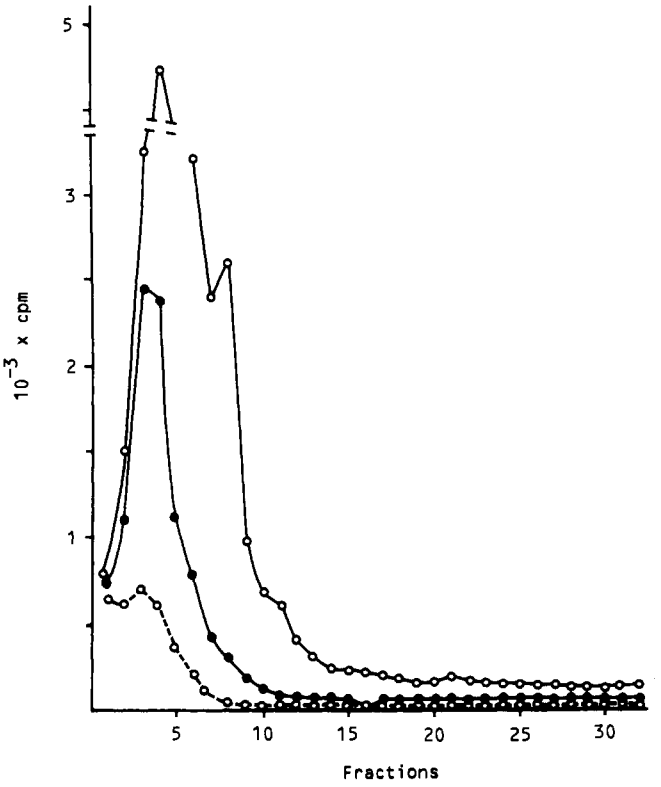

FIGURE 3: Alkaline sucrose gradients of DNA from nuclei labeled at lower temperatures. Conditions as described in the legend to Figure 1 except for incubation times and temperatures: (—○) 30 sec at 27°; (—●) 30 sec at 22°; (- - -○) 0 sec at 0°.

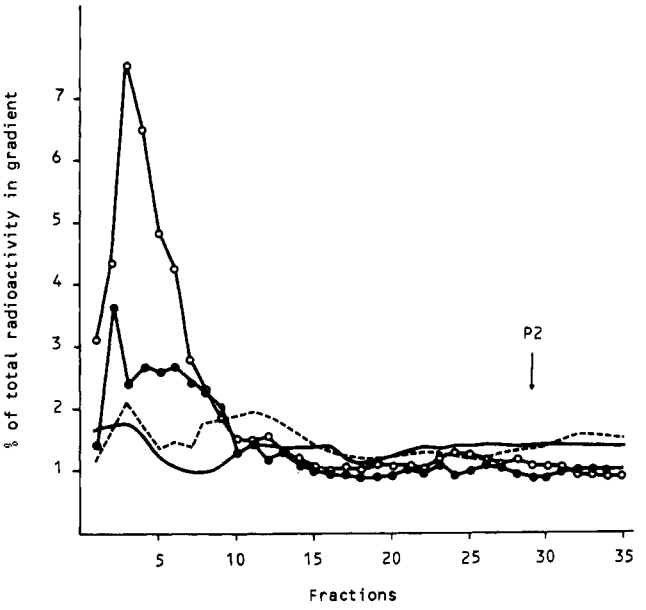

FIGURE 4: Alkaline sucrose gradients of DNA from nuclei labeled in short pulse-chase experiments. Nuclei were incubated without ribo- and deoxyribonucleotides for 60 sec at 37° and then labeled for 20 sec by the addition of ribonucleoside and deoxyribonucleoside triphosphates (—○) (final concentration of [^{3}H]TTP, 5 μM; specific activity, 30 mCi/μmol). A chase (115-fold excess of unlabeled TTP) of either 40 sec (—●), 130 sec (- - -), or 240 sec (—) was applied to identical aliquots. Lysis and centrifugation are as described in the caption to Figure 1. After the pulse the bottom fraction contained 33% of the total radioactivity recovered from the gradient. After the chase the bottom fraction contained 50% (40 sec), 50% (130 sec), and 53% (240 sec). The total radioactivity in each gradient varied from 45 × 10^3 to 58 × 10^3 cpm. The sedimentation of the P2 marker DNA is indicated.

in the relative amount of label in the primary DNA pieces.

The DNase sensitivity of the 2-4S DNA pieces was proved by pooling the appropriate fractions (fractions 2-7) from an alkaline sucrose gradient centrifugation of lysate from a 20-sec pulse experiment, dialyzing against 0.05 *M* Tris-HGl (pH 7.5) containing 10 m*M* $MgCl_2$, and digesting with pancreatic DNase (100 μg/ml for 45-180 min at 37°). A control sample was treated the same way and incubated with buffer. The primary DNA pieces were highly DNase sensitive; up to 83% of the radioactivity was rendered acid soluble. Another aliquot of the same pool (fractions 2-7) was dialyzed against 0.1 × SSC[1] and centrifuged to equilibrium in CsCl. The radioactive material banded at a density of 1.70 g/ml. No significant amount of radioactivity was found outside this peak.

Evidence for Ligation in Isolated Nuclei. To test whether ligation of primary DNA pieces takes place in isolated nuclei, pulse-chase experiments were performed. The nuclei were labeled for 20 sec, followed by a chase (115-fold excess of cold TTP) of different lengths (Figure 4). More than 50% of the label disappeared from the primary DNA pieces after a 40-sec chase. After a 130- or 240-sec chase most of the label was found in DNA larger than 30 S which sedimented to the bottom fraction under the centrifugation conditions used. When centrifuged under conditions where material with sedimentation coefficients smaller than 120 S did not sediment to the bottom (20,000 rpm/15 hr), a broad peak of 30-60S radioactive DNA was found both in a 20-sec pulse/40-sec chase experiment and in a 4-min pulse/25-min chase experiment (Figure 5). In both cases the incorporated label was preserved during the chase, excluding the possibility of specific losses of small DNA pieces.

Table I: Percent of Label Found in the Primary DNA Pieces after Different Pulse Lengths.[a]

Pulse Length (sec)	Incubation Temp (°C)	Total Acid Precipitable Radioact. in Gradient (cpm)	Label in Primary DNA Pieces (% of Total Label in DNA)
30	22	17,200	81
30	27	27,137	80
15	37	6,050	49
30	37	14,527	34
60	37	18,986	33
120	37	56,848	30
240	37	63,812	25
480	37	134,000	22
900	37	221,000	16

[a] The final concentration of [^{3}H]TTP was 5 μM and the final specific activity was 7.2 mCi/μmol at 37°. At 22 and 27° the final specific activity was 30 mCi/μmol.

These pulse-chase experiments thus showed that primary DNA pieces were ligated to fragments mainly larger than 30 S and no evidence was found for discrete classes of intermediate-size pieces between the primary DNA pieces (2-4 S) and the secondary DNA pieces (>30 S). (These secondary pieces probably correspond to the replication units.) Hence, the ligation process observed was best explained by assuming a direct stepwise ligation of primary DNA pieces to large (30-60 S) DNA fragments without any prior liga-

[1] Abbreviation used is: SSC, standard saline citrate in 150 m*M* NaCl in 15 m*M* sodium citrate.

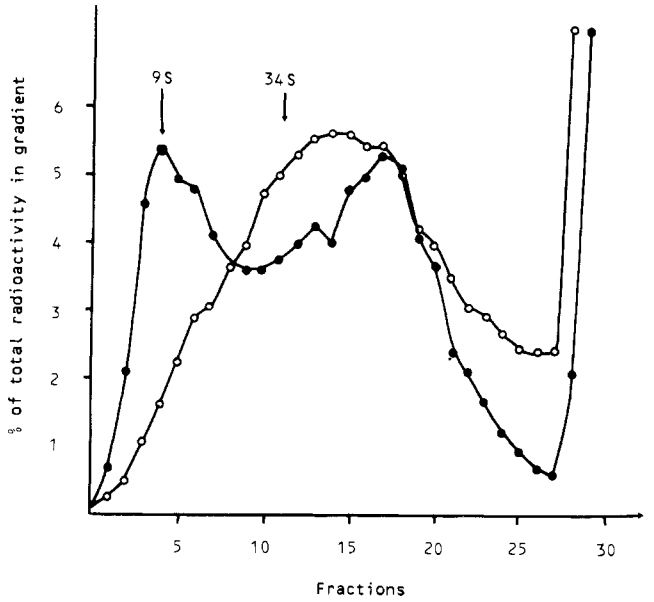

FIGURE 5: Alkaline sucrose gradients of DNA from nuclei labeled in long pulse-chase experiments. Nuclei were labeled for 4 min with [^{3}H]TTP as described for Figure 1 (—●) and then chased for 25 min (—○) (200-fold excess of unlabeled TTP). The nuclear lysate was centrifuged for 15 hr at 4° and 20,000 rpm. The bottom fraction contained 7% of the total radioactivity recovered.

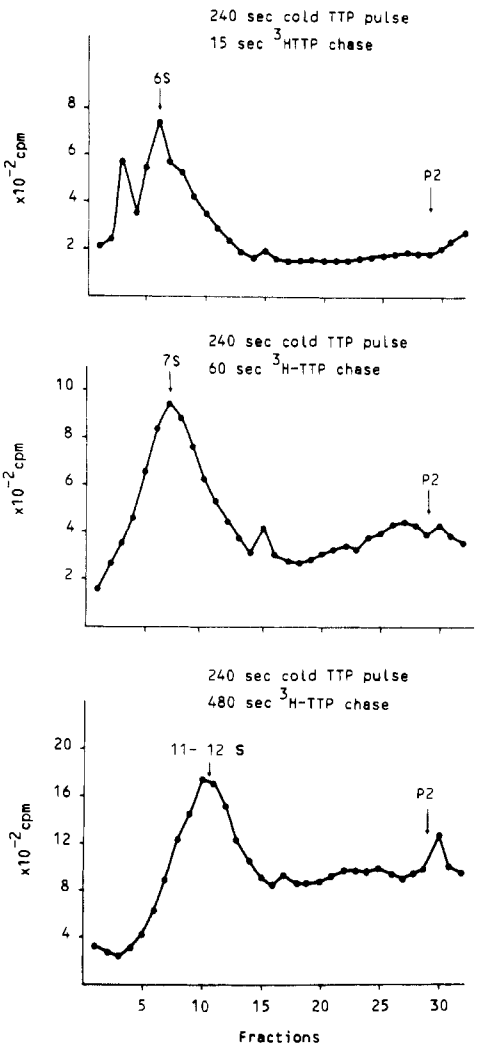

FIGURE 6: Alkaline sucrose gradients of DNA from nuclei labeled for 15-480 sec with [^{3}H]TTP (final concentration, 5 μM; specific activity, 7.2 mCi/μmol) after an initial 240-sec incubation with unlabeled TTP. Lysis and centrifugation as in Figure 1. The upper 30 fractions are shown.

tion occurring among the primary DNA pieces. The increase in size of the primary DNA pieces with increasing pulse length (Figure 1) does therefore probably not arise from limited ligation of such pieces, although this cannot be completely excluded. The bulk DNA in the alkaline sucrose gradients was estimated by the absorbancy at 260 and 280 nm and found to sediment to the bottom of the gradient when centrifuged at 20,000 rpm/15 hr at 4°. Nonreplicating DNA therefore was much larger than the secondary (30-60 S) DNA pieces.

Evidence for Initiation of Primary DNA Pieces in Vitro. In the pulse-chase experiments most of the label incorporated during the first 20 sec disappeared from the primary pieces within 130 sec. Primary DNA pieces initiated after that period were looked for by incubating the nuclei with a test mixture containing unlabeled TTP for 240 sec and subsequently adding [^{3}H]TTP (Figure 6). Label was incorporated into small DNA pieces of size 6-11 S, the size depending on the pulse length. The label appeared in 6S DNA when lysates from a 15-sec [^{3}H]TTP pulse after 240-sec preincubation without isotope were studied, whereas label appeared in 2S DNA when a 15-sec pulse only was used.

These experiments, together with the results of pulse-chase experiments (Figures 4 and 5), indicate that initiation of primary DNA pieces takes place in the nuclei in vitro.

Discussion

The present nuclear system has been shown to continue the replication of DNA at sites which were active in vivo (Krokan et al., 1975). In this paper we have demonstrated that the DNA synthesis in this system is discontinuous and that the primary DNA pieces made can be effectively chased into strands larger than 30 S. Taken together, the results illustrated in Figures 4 and 6 suggest that primary DNA pieces are initiated in vitro in this system. To our knowledge evidence for effective ligation of small pieces of nascent DNA in isolated nuclei has not been presented earlier. Recently, unsuccessful attempts to demonstrate ligation of DNA in isolated nuclei were reported by two groups (Friedman, 1974; Hershey and Taylor, 1974). In polyoma-infected mouse fibroblast nuclei, however, ligation of virus primary DNA pieces takes place (Winnacker et al., 1972; Magnusson et al., 1973). The reason for this discrepancy is not yet known and is most probably due to variations in the method of isolation of nuclei.

The increase in the size of the primary DNA pieces with increasing pulse length (Figure 1) was not due to a continuous elongation of the primary DNA pieces initially synthesized in vitro, nor was it caused by a ligation between such primary DNA pieces, since when a 20-sec [^{3}H]TTP pulse was followed by a chase with unlabeled TTP, most of the label disappeared from the top of the gradients and had a sedimentation coefficient >30 S. No labeled peak was observed in the 4-14S region, although there was a significant amount of label in this region.

The increase in length of the primary DNA pieces were therefore probably due to an alteration in the properties of the system during incubation. A possible explanation might be that the frequency of initiation of primary DNA pieces is reduced, allowing the synthesis of longer primary pieces. Olivera and Bonhoeffer (1972) found two classes of nascent

DNA in their *Escherichia coli* system. They suggested that the Okazaki pieces become longer as the ratio of initiation to elongation decreased. An analogous mechanism might determine the length of the primary DNA pieces which are initiated in the present system after the very first period of incubation. When the nuclei were pulse labeled at lower temperatures (Figure 3) to reduce the effective labeling time, more than 80% of the label was found as 2-4S pieces. The synthesis thus seems to be discontinuous both in the 5′→3′ and in the 3′→5′ directions. This is in agreement with recent results from intact cells (Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975; Goldstein and Rutman, 1973) but not in accordance with the results of Friedman (1974) and Hershey and Taylor (1974). Our results may also be caused by vastly different effects of lowering the temperature on the synthesis rate of the two sides of the fork. In intact cells the DNA synthesis rate is apparently more temperature sensitive than in isolated nuclei. A fivefold decrease was found in intact cells when the temperature was reduced from 37 to 26° (Huberman and Horwitz, 1973), whereas a reduction to 22° led only to a fourfold decrease of the DNA synthesis rate in isolated nuclei. In this nuclear system a 30-sec pulse at 27° corresponds to about a 15-sec pulse at 37°. A much larger part of the label (80% vs. 49%, Table I) was, however, found in the 2-4S fraction at the lower temperature. This may suggest that ligation is more temperature sensitive than initiation and elongation. Incubation at lower temperatures may be a convenient way to obtain larger quantities of primary DNA pieces.

The presence of acid-precipitable label in non-DNA material in the top region of alkaline sucrose gradients is no great problem when [^{3}H]TTP and isolated nuclei are used. It has been found, however, to be a problem when thymidine labeling of whole cells is used (J. A. Huberman, 1975, personal communication), and may illustrate one advantage of working with lysates of isolated nuclei as compared to lysates of whole cells provided that the system for isolation and incubation of nuclei is optimalized. A model for DNA synthesis in the present system of isolated nuclei compatible with the data in this report would be a discontinuous synthesis both in the 5′→3′ and in the 3′→5′ directions with primary DNA pieces of 2-4 S (about 50-200 nucleotides). These pieces are then ligated to secondary pieces of intermediate size (30-60 S) without any prior ligation among the primary pieces.

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